Diagnostically significant antigens of *Treponema pallidum* subsp. *pallidum*: identification, serological efficacy, and characterisation of the major antigenic determinants.

Thesis

UNRESTRICTED

Diagnostically significant antigens of *Treponema pallidum* subsp. *pallidum*; Identification, serological efficacy, and characterisation of the major antigenic determinants.

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Abstract

The antibody response to native antigens derived from *Treponema pallidum* (Nichols strain) was investigated by western blotting. In early primary disease the responses were most frequently to the major immunodominant antigen, TpN47, and the flagellar proteins. Strong responses to several other lipoproteins, especially TpN44.5, TpN17 and TpN15, were observed in all other disease stages. Generally, this confirmed the importance of TpN47, TpN44.5, TpN17, and TpN15 as diagnostically significant antigen.

Western blot analysis using recombinant proteins showed the four major lipoproteins, TpN47, TpN44.5, TpN17, and TpN15, to be important disease specific antigens, with TpN47 particularly reactive in early primary infections. TpN24-28 was found to be disease specific but only reactive with relatively few strongly positive sera, and so of relatively little diagnostic significance. Further analysis of the other recombinant proteins by enzyme immunoassay confirmed them as important diagnostic antigens, but no single recombinant protein was capable of detecting all cases of serologically confirmed syphilis. However, an enzyme immunoassay using a solid phase coated with a combination of all four major lipoproteins proved highly specific and showed improved sensitivity over a commercially available enzyme immunoassay based on native antigens.

Synthetic peptide epitope mapping studies of the four major lipoproteins showed that the antigenic sequences within these proteins were not simple linear epitopes. The complicated picture of several reactive regions across the proteins, each containing multiple epitopes, was typical of the type of responses seen when a substantial part of the antibody response is directed towards conformational epitopes.

Further analysis of the epitopes present within the four major lipoproteins using phage display isolated several long protein sequences which represented high affinity antibody binding motifs. The size of the sequences suggests that the retention by the solid phase is due either to conformational epitopes, or to a mixture of epitope types producing high affinity binding through multipoint attachment. The sequences identified have potential as antigens for use in diagnostic tests.

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Chapter 1

Introduction

Clinical disease

Syphilis is a systemic contagious disease of man cause by the spirochaete *Treponema pallidum* subsp. *pallidum*. It is a chronic disease characterised by multiple clinical stages, normally called primary, secondary and tertiary, interspersed by long periods of latent asymptomatic infection. The disease is usually sexually transmitted in adults. Less commonly, it can also be acquired by blood transfusion with blood from an infectious individual, needle sharing by intravenous drug users, and tattooing. Transplacental transmission to the fetus resulting in congenital syphilis remains a global problem, except in Northern Europe and the USA.

Due to the inability to cultivate the organism *in vitro*, serological diagnosis has become an important tool for both the diagnosis of infection at all stages of the disease, and the monitoring of treatment. The different manifestations of the disease offer unique challenges for serodiagnostic procedures, not fully countered by existing tests. This means there is considerable scope for improvements to serodiagnostic procedures to be gained by identification of diagnostically significant antigens. For antigens to be diagnostically significant they must be reactive at all stages of the disease. The following descriptions of the different manifestations of syphilis are taken from a selection of sources and are included for background information and to place the later discussions in context (Fitzgerald, 1981; Lukehart and Holmes, 1994; Tramont, 1995; Mindel *et al.*, 1989; Lossick and Kraus, 1991; Larsen *et al.*, 1990; Hutchinson and Hook, 1990; Thompson *et al.*, 1990; Thin, 1990; Sparling, 1990).

Untreated syphilis

T.pallidum can penetrate abraded skin or intact mucous membranes and spreads throughout the body via the bloodstream and lymphatics. 2 to 6 weeks later, depending on the size of the inoculum, a primary lesion develops at the site of contact and heals in a further 2 to 6 weeks. In a proportion of patients a secondary stage appears 6 weeks after the primary lesion has healed. In some patients the time between the primary lesion healing and the onset of the secondary stage can be prolonged to several months, in others these stages may overlap. In about 20% of cases infectious episodes occur during the following year. In the rest, the latent asymptomatic period follows which may persist for life in at least 60% of individuals. In 30 - 40% a third late destructive stage develops

involving the skin, mucous membranes, and bones. A more serious form may develop in some individuals also involving the CNS, aorta, and other internal organs.

The course of untreated syphilis has been documented in the Oslo study from 1890 to 1951. One thousand nine hundred and seventy-eight patients with early syphilis were left untreated. Gestland (1955) reanalysed the data from 1404 of the patients who were Norwegian residents of Oslo. Secondary syphilis relapse occurred in 25% of patients. Gummas (see below) were observed in 14.4% of males and 16.7% of females. Late neurosyphilis developed in 9.4% of males and 5.0% of females. Cardiovascular lesions were observed in 13.6% of males and 7.6% of females. A total of 23% of patients died as a direct result of syphilis. Serious late syphilitic complications were twice as common in males than females. There are several criticisms of this study. The study was terminated before all the patients had died and post-mortem examinations were not performed on all of the deceased. This means that late complications would have been underestimated. It is thought that some of the patients were probably suffering from non-venereal syphilis which was prevalent in Norway at the time. Also, some of the patients suffered reinfections, so that the study is not the course of one infection. Therefore, the study is more of historical interest rather than an estimate of the likely outcome of untreated disease.

Primary syphilis

After infection the organisms quickly spread throughout the body, but preferentially multiply at the site of entry, producing a small papule which rapidly ulcerates (Magnuson *et al.*, 1956). The ulcer, or chancre, is usually round or oval, and painless. It is not usually secondarily infected, which is a feature of all open syphilitic lesions. The chancre usually develops about 3 weeks after infection, although the incubation period between infection and development of the lesion can range from 10 to 90 days. The chancre may spontaneously heal after 2 to 8 weeks, although it may still be present during the secondary stage. Affected lymph nodes may be tender and contain numerous treponemes. Serous fluid from the chancre also contains large numbers of treponemes and is highly infectious.

Secondary syphilis

About 6 weeks after the primary chancre has disappeared the symptoms of secondary syphilis appear. By this stage of the disease, the organism has invaded every organ of the body and is

present in virtually all body fluids. This is demonstrated by the generalised and diverse nature of the symptoms seen, which include fever, headache, sore throat, arthralgias and anorexia. Headache, especially at night, is due to a low grade meningitis shown by pathological changes to the CSF. The most characteristic symptoms seen are a generalised rash, mucous patches and condylomata lata. Condylomata lata are probably the result of confluent moist papules covered by a white mucoid exudate. Mucous patches are painless greyish-white erosions forming circles and arcs, or 'snail-track' ulcers. Both of these ulcerative lesions are highly infectious, containing numerous treponemes. The rash usually covers the trunk and limbs, but appearance on the face, soles of the feet and palms of the hands is highly indicative of syphilis. It is usually accompanied by a generalised, painless, lymphadenopathy. More rarely symptoms include alopecia, laryngitis and hepatitis. All these lesions disappear spontaneously within 2 to 6 weeks and leave no evidence of infection behind. Infectious episodes can recur especially over the first year after disappearance of secondary symptoms.

Latent syphilis

If left untreated, the cutaneous lesions of secondary syphilis disappear and a period of latency follows. Latent syphilis is, by definition, an asymptomatic period with positive syphilis serology. For epidemiological purposes, latent disease of less than 2 years duration is termed early latent syphilis, otherwise it is classified as late latent syphilis. During the period of early latency the disease may relapse several times with the reappearance of symptoms of earlier disease. The patient remains infectious throughout this period and women continue to give birth to congenitally infected infants. Approximately 60% of patients remain latent for the rest of their lives with only weakly positive syphilis serology as the only indication of infection. Autopsy studies indicate that a proportion of these patients have subclinical infections, particularly of the cardiovascular system. The remaining 40% of patients will eventually develop clinical tertiary syphilis.

Tertiary syphilis

Benign and severe syphilitic lesions may develop 10 to 20 years after the initial infection. These lesions include, the formation of gummas in late benign syphilis, neurosyphilis, and cardiovascular syphilis.

Late benign syphilis

A proliferative or destructive inflammatory process called gumma formation provides the pathogenesis of late benign syphilis. Gummas are commonly observed in the skin and bones, but may also appear in the mucosa, viscera, muscles and eyes. The gumma is a chronic granulomatous lesion which is an intense inflammatory response to a few treponemes which impairs the function of the structure involved. It starts as a painless nodule which breaks down into one or several punched-out ulcers. Like lesions at other stages of the disease it is free from secondary infection. It heals slowly from the centre with distinctive pigmentation and a paper-thin scar. This pigmentation and scarring are quite characteristic and a useful aid to diagnosis. The most common sites involved are, the face, legs, buttocks, upper trunk, and scalp. Gummatous bone lesions can involve the tibia, fibula, palate, pharynx, and the nasal septum. They tend to be destructive, causing perforation of the hard palate and nasal septum leading to a saddle shaped nose. They also lead to a chronic hoarseness due to destruction of the epiglottis and larynx. The most serious lesion is the gummatous infiltration of the tongue which leads to a general swelling and a smooth red surface. Penicillin treatment has no effect on the progression of syphilitic glossitis at this late stage. The restricted blood supply eventually produces leucoplakia, necrotic white patches on the back of the tongue, which often become malignant.

Neurosyphilis

The cerebrospinal fluid is invaded by treponemes in the primary and secondary stages of the disease, which is reflected in changes to the cerebrospinal fluid. Asymptomatic neurosyphilis is the most common form of neurosyphilis and is defined as the absence of neurological symptoms with pathological changes to the cerebrospinal fluid. Changes may include positive syphilis serology, raised cell counts and protein levels. In patients with asymptomatic neurosyphilis the disease seldom progresses to neurosyphilis.

Symptomatic neurosyphilis can present at a variety of time intervals. Syphilitic meningitis may occur rapidly, while meningovascular syphilis can take a few years to present, and parenchymatous neurosyphilis is only seen several decades after the initial infection. In symptomatic neurosyphilis the lesions can appear anywhere in the central nervous system.

Syphilitic meningitis may develop within a year of infection. The symptoms resemble a viral meningitis which includes fever, stiff neck, and lymphocytosis. The more severe conditions of acute

syphilitic hydrocephalus, focal cerebral involvement, and cranial nerve palsy may appear in some patients.

Meningovascular syphilis is due to pathological changes in the medium and larger arteries of the brain and spinal cord. The vessel walls are gradually destroyed which induces thrombus formation, and causes narrowing of the vessel and eventual closure, resulting in a large cerebrovascular accident. The involvement of the spinal cord is a rare condition and manifests as weakness of the legs, which sometimes progresses to paralysis.

Parenchymatous neurosyphilis may be manifested as dementia paralytica or tabes dorsalis. Invasion of the cerebrum by *T.pallidum* causes meningoencephalitis and will result in a dementia paralytica after several decades, and finally in death. This condition may resemble almost any psychiatric or neurological disorder. Frequently symptoms include memory loss, diminishing intellectual functions, personality changes and dementia. Tabes dorsalis is very rare since the introduction of penicillin treatment.

Cardiovascular syphilis

In untreated syphilis cardiovascular symptoms develop after a period of 30 years. With modern antisyphilitic treatment very few patients ever have active infection for such a long period of time, so cardiovascular syphilis is rare. The cardiovascular system is not involved in the early stages of syphilis, so adequate treatment of the disease prevents syphilitic heart disease. Three major cardiovascular syndromes may occur, aortic aneurysm, aortic valve incompetence, and coronary ostial stenosis. The syphilitic aneurysm is the most common manifestation. The clinical symptoms are minor, consisting of mild chest pain and tambouric heart sounds. Aortic valve disease causes aortic regurgitation and a prominent second heart sound. The result may be enlargement and dilation of the left ventricle. Syphilitic disease of the coronary arteries results in narrowing of the vessels causing angina and sudden death due to extensive infarction.

Congenital syphilis

Congenital syphilis has always proved difficult to diagnose due to the clinical presentation. Diagnosis is often reliant on serological evidence, however serological testing is compromised by the presence of passively acquired maternal IgG antibodies in the foetal circulation. For this reason,

testing for the presence of treponemal specific foetal IgM antibodies has been adopted. Clearly, IgM responses must be considered when identifying diagnostically significant antigens.

The infection is usually transmitted to the fetus by transplacental passage of *T.pallidum* from an infected mother. An infant can also be infected at the time of delivery by contact with a genital lesion. The risk of congenital infection is directly related to the disease stage of the maternal infection (Wendel, 1988). The highest risk of transmission is during the primary and secondary stages, and as the disease progresses to the later latent stages the risk diminishes. The risk of fetal infection also increases as the pregnancy advances. Two characteristic syndromes can be identified, early and late congenital syphilis. Early congenital syphilis refers to those clinical manifestations that appear within the first 2 years of life. Those features that occur after 2 years and usually manifest near puberty constitute late syphilis.

Early congenital syphilis

A primary stage does not occur because the organisms directly infect the foetal circulation. The symptoms of congenital syphilis are similar to those found in secondary syphilis except the involvement is more systemic and the disease progression more rapid. The clinical manifestations of early congenital syphilis are a result of active infection and inflammation (Dorfman and Glaser, 1990; Sanchez *et al.*, 1983). Symptoms may be present at birth or delayed for several months if the infant remains untreated. Infants with congenital syphilis may have low birth weights, hepatosplenomegaly and generalised lymphadenopathy (Ingall *et al.*, 1990). Commonly, a haemolytic anaemia with thrombocytopaenia is present (Whitaker *et al.*, 1965). The reduction in platelet numbers is believed to be due to diminished survival mediated by circulating immune complexes. Rhinitis or 'snuffles' used to be common, but occurs less frequently today. The discharge is watery and contains a large number of spirochaetes, but becomes thick and purulent if left untreated. The infection can lead to involvement of the nasal cartilage resulting in perforation of the nasal septum and development of the saddle nose deformity (Schulz *et al.*, 1990). Involvement of the throat and upper airway may lead to hoarseness and laryngitis. The rash of congenital syphilis is initially oval and red, but then turns coppery brown with distinctive blisters containing numerous treponemes. The lesion is most prominent on the palms and soles of the feet. Mucous patches and condylomata lata may also occur. Skeletal lesions are commonly seen, which tend to be multiple and systemic, affecting the lower limbs most frequently (Cremin and Fisher, 1970).

Neurosyphilis is also quite common with features comparable to adult neurosyphilis. Acute syphilitic meningitis occurs, and if untreated can lead to chronic meningovascular syphilis which presents towards the end of the first year of life. A secondary nephrotic syndrome may develop which is due to deposition of immune complexes in the kidneys.

Late congenital syphilis

The clinical manifestations of late congenital syphilis are malformations that represent scars induced by the initial lesions of early congenital syphilis or reactions to persistent inflammation (Fiumara and Lesser, 1970; Rathbun, 1983). Infection of the tooth bud during late gestation causes a characteristic malformation known as Hutchinson's teeth. The upper central incisors are small, widely spaced, peg-shaped and notched with discoloured enamel (Ingall *et al.*, 1990). More diagnostic are malformations of the molars consisting of many small cusps instead of the usual four. They also show defective enamel and are prone to decay. Bone deformities are common, usually involving the skull and long bones. These include frontal bossing or squaring of the skull and bowing of the tibia. Involvement of the nasal septum may lead to bone destruction and result in a saddle nose deformity (Fiumara and Lesser, 1970). Linear scars or rhagades radiating from mucocutaneous junctions are the result of mucous patches and condylomata lata involving these areas. The eyes and ears may be affected resulting in interstitial keratitis and neural deafness due to cochlear degeneration. Interstitial keratitis, Hutchinson's teeth, and eighth nerve deafness comprise Hutchinson's triad which is specific for congenital syphilis (Ingall *et al.*, 1990). Neurosyphilis, dementia paralytica and tabes dorsalis may be found after puberty in untreated cases.

Congenital syphilis is effectively prevented by routine prenatal screening and penicillin treatment of infected women and their sexual partners. The development of the characteristic malformations can be prevented by treatment during pregnancy or within the first three months of life.

HIV co-infection

The course of syphilis is characterised by the failure of the immune system to completely eliminate the treponemes. This chronic infection and associated inflammatory reaction are responsible for many of the lesions seen. Clearly any factors which facilitate the persistence of the organisms may alter the course of the disease. HIV infection results in an immunosuppression and has been shown

to alter the natural course of syphilis (Johns *et al.*, 1987). However, the exact mechanism by which this happens is not understood. Confusing clinical symptoms and abnormal serological findings have been described (Radolf and Kaplan, 1988). Many authors have reported rapid progression to the late stages of syphilis and neurologic involvement even after treatment of primary or secondary syphilis (Dawson *et al.*, 1988; Musher, 1991; Kase *et al.*, 1988; Fernandez-Guerrero *et al.*, 1988). Neurosyphilis seldom appears in early syphilis in normal non HIV infected patients. Johns *et al.* reported four cases of neurosyphilis in HIV seropositive patients with active syphilis. One patient developed meningovascular syphilis within four months of infection, whereas in the non immunocompromised individual it usually develops 5 to 10 years after the initial infection. The effect of maternal HIV infection on the likelihood of congenital syphilis developing is not fully understood. The immune dysfunction is likely to allow greater proliferation of the treponemes and a higher rate of infection. Infants born to HIV infected mothers with untreated early syphilis are more likely to show symptoms of congenital syphilis than those born to HIV seronegative mothers with early syphilis (Sánchez *et al.*, 1990). This suggests that the disease is perhaps more severe which is a similar picture to that seen with HIV infected adults. Concurrent HIV and syphilis infections raise new considerations related to diagnosis, treatment, and the progression of the disease. The atypically weak or non-existent reactivity in standard serological tests, and the absence of obvious symptoms in some cases has profound implications for the detection of infection. The changes in the immune response present in this group of patients have implications for both, the level of sensitivity required from serodiagnostic tests, and potential changes to the antigens that the antibody response is directed towards. This makes this an important group of patients to consider when developing and evaluating new serological tests.

Treatment

Penicillin treatment was introduced in 1944 and remains the treatment of choice. Penicillin inhibits bacterial cell wall construction and therefore only destroys actively growing bacteria. The replication time of *T.pallidum* is about 30 hours (Cumberland and Turner, 1949). Generally, treatment regimes aim to produce a high penicillin concentration and maintain it over a prolonged period of time. The regimes are extended for advanced disease to allow the drug to permeate all body tissues and completely eliminate the organisms. There are a wide variety of different treatment regimes described, all producing similar results, suggesting a fair degree of latitude is permissible. Effective

treatment is achieved if the concentration of penicillin in the serum is maintained above 0.03 U/mL for 8 to 10 days, and the troughs in the concentration should not exceed 15 hours (Mohr *et al.*, 1976). The World Health Organisation recommend treatment with 1.2 MU of procaine penicillin G daily for 10 days for early syphilis, and an increase in the duration of the programme to 15-20 days for late syphilis. This regime was used successfully in the mass treatment campaign against non-venereal syphilis. An alternative treatment that is effective at most stages of syphilis is a single injection of the long-acting benzathine penicillin (2.4MU). However, it is unsuitable for treating neurosyphilis as it does not reach the CSF (Polnikorn *et al.*, 1980). Failure to reach therapeutic levels in the CSF also preclude its use for the treatment of congenital syphilis and HIV seropositive patients. This regime is most suitable where the patient can only attend the clinic once and is unable or unlikely to continue the treatment elsewhere. In the event of penicillin allergy, tetracycline/doxycycline and erythromycin or its newer relatives are the alternative drugs. All treponemal infections are unaffected by sulphonamides, rifampicin, and quinolones at clinical doses.

Syphilis responds well to antibiotic therapy and is easily cured in most cases. The monitoring of treatment and confirmation of cure can not be accomplished by conventional methods because the organism can not be cultivated. However, serological tests have been used for this function, due to the finding that the cardiolipin response detected by nontreponemal test (see below) diminishes on successful treatment. Conversely, specific anti-treponemal antibodies remain present for life in many cases, which make all currently available treponemal tests (see below) unsuitable for treatment monitoring. The identification of diagnostically significant antigens that become non reactive after treatment would be of considerable benefit to the field of syphilis serology.

Non-venereal treponematoses

Other spirochaetes of the genus Treponema cause a group of chronic granulomatous diseases which include yaws, non venereal endemic syphilis or bejel, and pinta. In sequence, the causative organisms are; *T.pallidum* subsp. *pertenue*, *T.pallidum* subsp. *endemicum*, and *T.carateum*. These organisms are morphologically indistinguishable from *T.pallidum* subsp. *pallidum*, and share common antigens resulting in a degree of crossimmunity. The differences between the various subspecies are discussed more fully later. No laboratory test has yet been devised that can distinguish these organisms from each other, or from *T.pallidum* subsp. *pallidum*. The identification

of antigens capable of making this distinction would be very useful but, in view of the extensive DNA homology (see below) shown by these organisms, this is extremely unlikely. They are primarily categorised as separate organisms due to differences in the clinical pictures produced. The diseases, and hence the organisms, are distinguished by the clinical appearance and anatomical site of the lesions, the mode of transmission, the age and geographical location of the individual.

Yaws is transmitted by direct contact with an infectious lesion or by fingers contaminated with lesion exudate. Yaws transmission is enhanced by crowded environments with poor sanitation and personal hygiene (Engelkens *et al.*, 1991a). The disease is usually acquired in childhood between the ages of 5 and 15. There is no racial or sex difference between those who become infected and in endemic areas more than 80% of the population are infected (Benenson, 1990a). Climatic conditions affect the type of yaws lesion and its transmission. In warm humid areas the lesion proliferates and contains many treponemes, whereas in dry, arid regions the lesions tend to be small or absent, after the primary lesion disappears.

Endemic syphilis is also transmitted by non-venereal contact among children. In contrast to yaws, transmission of infection by contaminated drinking vessels may be more common than by direct contact with infectious lesions (Benenson, 1990b; Engelkens *et al.*, 1991b). The disease tends to be familial with spread of infection from children to adults rather than to the general community. Endemic syphilis lesions are virtually indistinguishable from early yaws and the two diseases may occur at different times in the same population but not in the same person.

Several variants of endemic syphilis are recognised by their geographical location showing different disease expression. *Sibbens* of Scotland, *radesyge* of Norway, and *skerjevo* of Yugoslavia are now extinct, while *Njovera* or *dichuchwa* of Africa is still found in small pockets (Antal and Causse, 1985). *Bejel* of the Eastern Mediterranean is the only variant still prevalent. It is found in semi-nomadic people living in the Saharan regions of Africa. Improved living standards, better medical care, and mass penicillin treatment campaigns have greatly reduced its prevalence (Meheus and Antal, 1992)

Pinta is restricted to the western hemisphere and is found only in remote parts of Southern Mexico and small regions of Columbia, Peru, Ecuador, and Venezuela (Benenson, 1990c). Treatment programmes have greatly reduced the prevalence of pinta.

Laboratory diagnosis

T.pallidum can not be routinely cultured *in vitro* (Fieldsteel *et al.*, 1981., Norris, 1982.) or stained with simple laboratory stains (Swisher, 1987), therefore serological methods have formed the basis for the identification of syphilis infection. Many tests have been developed over the years, but most have arisen as modifications of previous tests in an attempt to rectify deficiencies. These tests generally use crude antigen preparations and there is a poor understanding of the underlying principles of some of these tests, particularly the cardiolipin response. An historical perspective of the sequence of diagnostic test evolution gives a useful insight into the type of problems encountered and how they were overcome. As current tests are derived from these historical methods; many of the problems described still exist today.

History of syphilis testing

Treponema pallidum subsp. *pallidum* was first associated with syphilis by Schaudin and Hoffman in 1905 using a modified Giemsa stain to examine lesion material. In 1909 Coles described the use of dark field microscopy to identify the organism. This method remains in use today for the examination of the exudate from lesions present in early syphilis. Many serological methods exist and can be categorised into two types; nontreponemal tests and treponemal tests.

Nontreponemal tests

In 1906, Wassermann adapted the complement fixation test to detect antibodies present during syphilis using an antigen derived from the liver of newborn infants who had died of congenital syphilis. This test was thought to be specific but Landsteiner showed that extracts of beef heart worked equally well. These complement fixation tests required complicated reagents and were quite time consuming to perform. A major advance was made in 1922 when Khan developed a macroscopically read flocculation test that did not require complement and took only a few hours to perform. Many derivations of the Khan flocculation test were developed but they all suffered from the same limitations. The antigens used were crude preparations which were poorly standardised, therefore results were difficult to compare between different testing occasions and different laboratories. This was solved in 1941 when Pangborn isolated from beef heart the active antigenic component, cardiolipin. Cardiolipin, in combination with cholesterol and lecithin, produces a specific antigen for the detection of antibodies of the so-called 'reagin' type found in active syphilis. This

antigen had major advantages over the previous crude extracts. It could be standardised both chemically and serologically, thus ensuring reproducibility of results between test runs and between different laboratories. These purified antigens allowed new microflocculation tests to be developed such as the Venereal Disease Research Laboratory (VDRL) test (Harris *et al.*, 1946). These tests yielded reproducible results, could be performed rapidly, and gave acceptable levels of sensitivity and specificity. The VDRL could only be performed on serum specimens because of the need to heat the sample before testing. To overcome this, a modification was developed where EDTA and choline chloride were added to stabilise the antigen and improve its reactivity. The resulting unheated serum reagin (USR) test (Portnoy *et al.*, 1961), did not need the sample to be heated and could use plasma as well. The rapid plasma reagin (RPR) teardrop card test (Portnoy *et al.*, 1957, 1961) was developed as a field test using charcoal particles to improve visualisation of the reaction. The test was performed on plasma from blood obtained by finger prick on a card rocked by hand. A slight modification was developed, more suitable for mass screening in a laboratory setting (Falcone *et al.*, 1964). This test is known as the RPR 18mm circle card test and is slightly more sensitive than the field test version. An automated version of the RPR was developed (McGrew *et al.*, 1968) which was particularly applicable to situations where large numbers of specimens have to be tested, like blood transfusion centres and public health laboratories. It used a continuous flow analyser to mix the reactants and the results were deposited on to a paper strip for interpretation. A final modification to the RPR test, this time involving the antigen and not the protocol, produced the toluidine red unheated serum test (TRUST) (Pettit *et al.*, 1983). The charcoal used to visualise the reaction was replaced by toluidine red particles. The VDRL, USR, TRUST, and RPR tests, are all still in use today, however the RPR, in all of its guises, is probably the most popular test.

Treponemal tests

Nontreponemal tests were found to suffer from nonspecific or false-positive reactions (see below), so a search for a specific serological test for syphilis was undertaken using antigens derived from the treponeme itself. The first attempt was the *T.pallidum* immobilisation (TPI) test which used dark field microscopy to look for the ability of a serum specimen, in the presence of complement, to immobilise live treponemes. The test has faded from use because it was extremely complicated to perform and required a supply of live *T.pallidum*. Much simpler and less expensive procedures have made it redundant. The Reiter treponeme, *T.phagedenis*, can be easily cultivated *in vitro* and was

thought to be a substitute for *T.pallidum* with identical antigenic specificity. Complement fixation tests using Reiter treponeme antigen were developed but a problem of significant false positive reactions and the inherent complexities of the procedure heralded their demise. The first significant development in treponemal serology was the fluorescent treponemal antibody (FTA) test (Deacon *et al.*, 1957). This detected specific treponemal antibodies using killed *T.pallidum* (Nichols strain) fixed to a glass slide and a fluorescently labelled anti-human immunoglobulin reagent to visualise the bound antibodies from the specimen. The test was found to suffer from false positive reactions and so a method using an increased specimen dilution of 1:200 was developed, the FTA-200 (Deacon *et al.*, 1960). However, although highly specific, the lower specimen concentration used made this test insensitive. The problem was solved by the inclusion of an absorbing specimen diluent containing an extract prepared by sonicating cultures of *T.phagedenis* (Hunter *et al.*, 1964). The diluent removed cross reacting antibodies thereby improving both the sensitivity and specificity of the original test. The modified method came to be known as the FTA absorption (FTA-ABS) test and is used today as a confirmatory test for syphilis. The FTA-ABS test, although specific, was not suitable for testing large numbers of specimens because of its complexity and low throughput. So, a quick and inexpensive treponemal test suitable for mass screening was still sought.

Rathlev (1965, 1967) was the first to successfully apply haemagglutination techniques to the serodiagnosis of syphilis. In the test, tanned sheep red blood cells coated with sonicated *T.pallidum* would agglutinate in the presence of treponemal antibodies in a patient's serum. Modifications to the basic procedure followed that were designed to improve the sensitivity and specificity of the test. A new reaction medium improved the reactivity and the inclusion of an absorbing diluent, similar to the one used in the FTA-ABS test, reduced the number of false positive reactions (Tomizawa and Kasamatsu, 1966, 1969). This final form of the test is known as the *T.pallidum* haemagglutination assay (TPHA). The test was originally performed in a tube format and so used relatively large volumes of reagents. A version was developed in a microplate format that used much smaller quantities of reagents, the microhaemagglutination assay for antibodies to *T.pallidum* (MHA-TP). The TPHA and the MHA-TP tests are both widely used today as treponemal screening tests. Various commercial versions of the tests have been developed each with its own features. The main variation, in some tests, has been the replacement of sheep red blood cells by avian red blood cells to increase the sedimentation rate and hence reduce the assay time. This principle is used in

the PK-*T.pallidum* (PK-TP) test, an automated version principally for blood donor screening on the Olympus PK7100 analyser.

Commonly used diagnostic tests

Over the years there have been many tests for syphilis developed, but as can be seen from the review above many are simply modifications of the core principle. Many of these tests are still in widespread use today. Even though each has its limitations, testing strategies have been adopted to minimise their deficiencies. This means that these tests tend to persist even though better methods are available using more modern techniques.

Direct microscopic examination

Syphilis can be diagnosed by direct visualisation of the organism by dark field microscopy. The technique is most applicable during primary and secondary syphilis, and relapsing episodes when mucocutaneous lesions are present containing numerous treponemes. *T.pallidum* can be identified in serous fluid taken from a lesion by their characteristic morphology and motility. However, *T.pallidum* subsp. *pallidum* can not be distinguished by visualisation from *T.pallidum* subsp. *pertenue*, *T.pallidum* subsp. *endemicum*, and *T.carateum*. Commensal spirochaetes are found as part of the normal flora of the genital and rectal mucosal surfaces, and are present in the mouth. *T.refringens* and *T.denticola* can be easily confused with *T.pallidum* (Creighton, 1990). Therefore, dark field microscopy may not provide reliable diagnosis in the case of non-penile lesions. Dark field microscopy may also be performed on aspirates taken from enlarged lymph nodes in cases where external lesions are absent. If non-pathogenic treponemes can be ruled out, the demonstration of organisms by dark field microscopy is diagnostic of syphilis. The sensitivity of dark field examination approaches 80%. Problems associated with dark field microscopy are that it must be performed immediately as it requires live organisms so the specimens can not be transported. This means that the test can only be performed in clinics with appropriately trained staff and the necessary equipment. Additionally, there is the risk involved in examining potentially infectious specimens, possibly containing HIV. A method that could identify *T.pallidum* in dry or fixed slides would offer considerable advantages. The specimens could be transported to a specialist laboratory for examination and much of the infection risk could be eliminated.

The direct fluorescent antibody (DFA-TP) test is an enhanced direct detection method that uses fluorescently labelled antibodies to distinguish non-pathogenic treponemes from pathogenic treponemes in fixed smears. As the test uses fixed specimens, and does not require the organisms to be motile, therefore it is not as reliant on specimen condition as dark field microscopy. The original test used fluorescently labelled Immunoglobulin G (IgG) prepared from either human sera or immunised rabbits (Kellogg and Mothershed, 1969), but this proved to still give specificity problems. Improvements in specificity were achieved by using a fluorescently labelled monoclonal antibody specific for TpN47, the pathogen specific immunodominant antigen of *T.pallidum* (Ito *et al.*, 1992). This test was found to be 100% specific and sensitive, correctly identifying 30 specimens with syphilis from 31 specimens without. In contrast, dark field microscopy gave a sensitivity of 97% and a specificity of 77% (Hook *et al.*, 1985). This suggests that the DFA-TP test is slightly more sensitive than dark field, but considerably more specific. However, in another study consisting of twice the number of patients, the two tests gave relatively similar sensitivities, and both gave specificities of 100%. Dark field showed a slightly better sensitivity than DFA-TP, 79% compared to 73% (Romanwski *et al.*, 1987). This second study shows the two tests to be fairly comparable in terms of sensitivity but DFA-TP is potentially more specific. Considering the practical advantages of the DFA-TP test over dark field microscopy it may become the method of choice for examining lesion material. The test has been adapted to detect *T.pallidum* in tissue sections (Ito *et al.*, 1991), so a combination of the DFA-TP test and histological staining can be used to examine biopsy and autopsy material.

In the absence of *in vitro* culture, these direct detection methods are the only way to confirm the diagnosis of syphilis. However, they are only viable during the stages of the disease when lesions containing treponemes are present. It is for this reason that the use of these methods persists in specialised clinics. During the stages of syphilis when lesions are absent serological tests form the main diagnostic tool.

Nontreponemal tests

Four nontreponemal tests are in widespread use; the VDRL test, the USR test, the TRUST, and the RPR test. These tests are similar in format and based on a common antigen which is composed of an alcoholic solution containing cardiolipin, cholesterol, and sufficient purified lecithin to produce standardised reactivity and enable emulsification. They differ in minor features only, specimen type,

the matrix used to visualise the reaction, and the method of reading the result. The VDRL can not be performed on plasma as the specimen has to be heat inactivated before testing, but the other tests can be performed on serum or plasma. The VDRL and the USR must be read microscopically as they do not contain anything to help visualise the flocculation of the antigen preparation. In contrast, the RPR and the TRUST tests contain coloured particles which become trapped in the matrix as the antigen flocculates. The RPR test uses particles of carbon, whereas the TRUST uses toluidine red dye particles. The inclusion of visible particles allows the tests to be read macroscopically. All four tests are performed on plastic coated cards or glass slides onto which rings have been printed. Standardised amounts of undiluted serum and antigen suspension are mixed together and spread out within the ring. The card is then mechanically or manually rotated under humid conditions. When serum contains anti-cardiolipin antibodies, flocculation occurs which can be seen by the trapped carbon particles in the aggregates or read microscopically.

The test results are read immediately after rotation and scored as reactive or non-reactive. False negative results, in specimens with high antibody titres, can occur due to the excess antibody preventing agglutination. This is known as the prozone phenomenon. This can be overcome by testing the specimen at a range of dilutions. Semi-quantitative testing by serially diluting the serum can be used to monitor the change in titre as a result of treatment. The result is expressed as the reciprocal of the highest reactive dilution.

These nontreponemal tests measure IgM and IgG antibodies to lipoidal material released from damaged host cells as well as lipoprotein-like material (Matthews *et al.*, 1979) and possibly cardiolipin released from the treponemes. The anti-lipoidal antibodies are produced not only as a result of treponemal disease, but also in other diseases where tissue damage occurs (Catteral, 1972). Acute false positive reactions have been associated with many types of viral infections, malaria, immunizations, and pregnancy (Grossman and Peery, 1969; Jaffe and Musher, 1990; Salo *et al.*, 1967; Sparling, 1971). Chronic false positive reactions have been associated with systemic lupus erythrematosus, malignancy, narcotic addiction, leprosy, and old age (Jaffe and Musher, 1990; Tuffanelli, 1966). Therefore, a reactive nontreponemal test does not confirm *T.pallidum* infection.

These tests are used as screening tests because they are widely available, inexpensive and convenient to perform. Their disadvantages are a lack of sensitivity in early primary infections and in

late syphilis, and the potential to give false negatives due to a prozone phenomenon. Their main advantage, and one of the reasons their use persists, is that they become non reactive after successful treatment. Therefore, they are useful tools for monitoring the progress of treatment regimens. Positive results are usually confirmed by retesting with a treponemal test.

Treponemal tests

Due to nonspecific and false-positive results found with nontreponemal tests many different treponemal tests have been developed over the years. They are based around specific antigens associated with treponemes and detect antibodies formed by the host to *T.pallidum* infection. The FTA-ABS test is an indirect fluorescent antibody technique. The patient's serum is diluted in an adsorbing diluent, the main component of which is an extract from cultures of the non-pathogenic Reiter treponeme. This diluent is designed to remove group treponemal antibodies produced in some individuals in response to non-pathogenic treponemes. The diluted serum is dispensed on to a microscope slide to which *T.pallidum* have been fixed. If anti-treponemal antibodies are present they bind to the immobilised *T.pallidum*. FITC labelled anti-human IgG is added and binds to the patient's antibodies resulting in FITC bound to treponemes, which can be visualised by fluorescent microscopy. Most laboratories use commercial kits for performing FTA-ABS tests and studies have shown large discrepancies between the performance of different kits (Beebe and Nouri, 1984; Chronas *et al.*, 1992). A modification of the FTA-ABS test is the FTA-ABS double staining test which employs a tetramethlyrhodamine isothiocyanate labelled anti-human IgG and a counterstain of FITC-labelled anti-*T.pallidum* conjugate. The counterstain was developed to eliminate the need to locate the treponemes by dark field. Counterstaining the organisms ensures that a non-reactive result is due to absence of antibodies and not absence of treponemes. These tests are complex systems requiring careful balancing of reagents, experienced operators and accurate setup of equipment to achieve consistent results. The specificity is not absolute, it ranges from 92% to 99% (Hunter *et al.*, 1986) and has a false positive rate of around 1% in normal individuals (Luger, 1988). These tests are used exclusively to confirm a positive nontreponemal or haemagglutination test.

Haemagglutination tests offer a relatively simple quick procedure to detect specific treponemal antibodies. The MHA-TP is probably the most widely used test. The basic principle of all the haemagglutination tests is similar. Formalised, tanned sheep or avian red blood cells are coated with antigens derived from sonicated *T.pallidum* (Nichols strain). The suspension of coated

erythrocytes is mixed with serum diluted in an adsorbing diluent containing Reiter antigen and other adsorbents. The Reiter antigen is used to remove group specific treponemal antibodies and thereby reduce the incidence of false positive reactions. If anti-treponemal antibodies are present in the specimen they bind to the sensitised erythrocytes and cause agglutination. A reactive result is seen by the formation of a smooth mat of cells on the bottom of the well. This is contrasted by a negative reaction where the cells settle to form a small round button in the bottom of the well. Specimens are tested in parallel with uncoated control cells to control against false reactions caused by anti-species antibodies. The test is recorded as reactive only if the coated cells are reactive and the uncoated cells are non-reactive. The haemagglutination assays detect *T.pallidum* specific IgM and IgG antibodies. However, despite the complicated adsorbing diluents false reactions still occur in a small number of specimens. The haemagglutination tests are suitable for testing large numbers of samples because they are easy to set up and simple to read. They can be used for screening or confirmation of a positive nontreponemal test. The test can be performed quantitatively by testing dilutions of the specimen, but no relationship has been identified between titre and the stage of the disease or its progression. The test remains positive even after treatment, sometimes for life, so it is not suitable for monitoring treatment. Positive haemagglutination test results are usually confirmed using a fluorescent antibody test.

Other tests

Enzyme immunoassay, western blotting, and more recently DNA based methods, have had a major impact on the field of infectious disease diagnosis. However, their adoption for the diagnosis of syphilis has been slow and the older tests are still in widespread use. These tests offer distinct advantages over the commonly used cardiolipin tests and haemagglutination assays, and are likely to eventually become more widely adopted.

Enzyme immunoassays

Enzyme immunoassays have been used to great effect for the serodiagnosis of infectious diseases and have provided numerous benefits in many areas. A large number of different assay designs exist and a full description of them all is outside the scope of this thesis. Briefly however, the indirect enzyme immunoassay is based on a solid-phase coated with a source of antigen. The solid-phase can be the inside surface of a test tube, microplate well, or the outside of a bead. The diluted specimen is incubated with the solid-phase to allow any specific antibodies to bind. Unbound

material is removed by sequential washes in a buffer containing detergent. The bound antibodies are detected by incubating the solid-phase with a dilution of an enzyme labelled anti-immunoglobulin antibody. Again, unbound material is removed by sequential washes. The bound enzyme is detected by the addition of a substrate which is converted from a colourless state to a coloured one in the presence of the enzyme. The enzyme reaction is stopped by destroying or inhibiting the enzyme and the coloured product measured spectrophotometrically. So, the quantity of specific antibody in the original specimen is proportional to the amount of coloured product produced in the enzyme reaction. Since the first report of enzyme immunoassay being used for the serodiagnosis of syphilis (Veldkamp and Visser, 1975), several other tests have been reported which used sonicated *T.pallidum* (Nichols strain) as the solid-phase antigen (Pope *et al.*, 1982; Farshy *et al.*, 1984; Hunter *et al.*, 1982; Farshy *et al.*, 1985; Codd *et al.*, 1988). Enzyme immunoassays that use components of the nonpathogenic Reiter treponeme (Pedersen *et al.*, 1982) and modified cardiolipin antigen (Pedersen *et al.*, 1987; White and Fuller, 1989) as antigen sources have also been reported. All the authors report respectable sensitivity and specificity figures for these tests, which are no worse than established tests. Additionally, these tests offer practical advantages, in terms of specimen processing and result reporting, compared to the widely used nontreponemal and treponemal tests. On this basis alone, these tests offer benefits over existing serological tests for syphilis. The disadvantages of this type of method are their complexity and the specialised equipment needed to perform them. They are best suited to processing large numbers of specimens rather than single tests. As the reagents require careful balancing to get optimal performance and the assay requires quality control to maintain consistent performance, these methods are best suited to large specialised laboratories. Most laboratories do not have the facilities to develop and maintain their own in-house methods so most enzyme immunoassays used are commercial kits. In-house assays tend to be less well standardised resulting in variable performance and are not extensively characterised, or approved by regulatory authorities. Commercial kits come and go, but several have been around for many years and so have established performance data.

The Syphilis Bio-EnzaBead test is an indirect immunoassay format with the solid phase consisting of ferrous metal beads coated with sonicated *T.pallidum*. The sequential steps of the procedure are performed by transferring the beads to fresh wells using a magnetic transfer device. It has been

evaluated by several groups (Stevens *et al.*, 1985; Moyer *et al.*, 1987; Burdash *et al.*, 1987; Larsen *et al.*, 1987), but only the last group distinguished the specimens on the basis of disease stage. They found an overall sensitivity of 94.7% and FTA-ABS gave 98.1%. The sensitivity with FTA-ABS positive primary and secondary syphilis was 100% and in early latent and late latent syphilis it was 96.4% and 84.6%, respectively. Another widely evaluated commercial test is the Captia® Syphilis-G test, an indirect immunoassay in a microplate format with wells coated with sonicated *T.pallidum* antigen. Young *et al.* (1989) reported an overall sensitivity of 98.4% and a specificity of 99.3%, while Lefevre *et al.* (1990) found an overall sensitivity of 98.3%.

Enzyme immunoassay has been used to detect IgM antibodies, which are of particular significance in early infection and congenital syphilis. The only other way to detect IgM antibodies is the FTA-ABS 19S IgM test which is extremely difficult to perform reliably and is not available in kit form. All IgM tests require the separation of IgM from IgG to remove competition from disease specific IgG antibodies, the presence of which could cause false negative reactions and the removal of rheumatoid factor which can cause false positive reactions. Many enzyme immunoassays have been developed that detect specific IgM using an anti-IgM reagent and an adsorbing diluent to remove the IgG and the rheumatoid factor. This strategy is only of limited success due to the difficulties encountered in ensuring that the adsorption step is complete.

A more successful format is the IgM-capture principle. The solid phase is coated with an anti-IgM antibody which captures a sample of the IgM present in the specimen. Disease specific IgM is detected using *T.pallidum* antigen labelled with a monoclonal antibody conjugated to an enzyme. After unbound material has been removed by sequential washes, the bound enzyme is detected by the addition of a substrate. The advantage of this format is that it is a much simpler procedure as there is no need for a separation stage and there is no interference from rheumatoid factor. The disadvantages are that it requires careful balancing of the reagents and the use of high concentrations of antigen to achieve the performance. This makes the test complicated to develop and maintain, and costly to produce. Again, it is probably best suited to large laboratories with resources to maintain and control the test, however most laboratories use commercial kits. A kit that has been widely evaluated is the Captia® Syphilis-M test. Several evaluations have been performed with reasonable agreement between the studies, although the numbers of specimens tested was low (Lefevre *et al.*, 1990; Ijsselmuiden *et al.*, 1989b). The sensitivity was found to be

highest in early primary syphilis at around 90% and decreased with disease progression. Both studies found the test to be positive in all cases of congenital syphilis that were examined. Another study that evaluated it specifically in relation to congenital syphilis (Stoll *et al.*, 1993) found it more sensitive than FTA-ABS 19S at detecting probable cases.

Western blotting

Western blotting has been used widely for infectious disease diagnosis. The high sensitivity and specificity it offers make it an ideal confirmatory test. It has been particularly effective for the confirmation of human immunodeficiency virus infection. The test is performed by first electrophoretically separating an extracted sample of the organism on an SDS polyacrylamide gel. The gel is removed from the apparatus and a sheet of nitrocellulose is laid on top. The protein bands are electrophoretically transferred to the membrane, which is cut into separate longitudinal strips for testing against individual specimens. A strip is incubated with diluted patient's serum and any specific antibodies bind to the proteins immobilised on the membrane. The strip is washed several times to remove unbound material before being incubated with an anti-human immunoglobulin antibody conjugated to an enzyme. The conjugate will bind to any antibodies attached to the proteins on the membrane. After a series of washes to remove unbound conjugate, the remaining material is detected using an enzyme substrate which produces an insoluble coloured product. The coloured product precipitates within the membrane causing the reactive proteins to be seen as dark lines on the membrane.

The first report of the use of western blotting for the diagnosis of syphilis was in 1985 (Hensel *et al.*, 1985). The problem with western blotting is that the result is not in a quantitative form, so the interpretation can be subjective and relies on the correct identification of key protein bands. Until 1993 when the nomenclature was standardised (Norris *et al.*, 1993), individual researchers referred to proteins by their own designations, so it was difficult to develop a consensus definition of which reactive bands constituted a positive result. However, all reports support its value as a confirmatory test. Dettori *et al.* (1989) found reactivity to the 15kDa protein in 97% of 110 confirmed cases of syphilis. The cases that were negative were two seronegative early primary infections and a case of late latent treated syphilis. This shows the high sensitivity of the test as these cases are extremely difficult cases to detect by conventional means. The specificity was shown by the absence of antibodies in 47 patients with false positive syphilis tests and 121 normal blood donors. In another

study the criteria for a positive blot were that at least 3 of the 4 antigens, 47, 44.5, 17 and 15kDa, should be reactive (Byrne *et al.*, 1992). They found an overall sensitivity of 91.7% and a specificity of 100% in clinical defined specimens. The test was very sensitive in early primary infection, detecting 37 of 40 cases (93%). This may have been due to the use of a conjugate specific for both IgG and IgM antibodies. Generally, there is a consensus that the significant proteins are those identified by these researchers, but the exact number of the bands that have to be reactive to qualify as a positive blot remains a contentious issue.

Western blotting has also been applied to detecting IgM antibodies, particularly in congenital syphilis. The test is more complicated than that described above; it requires the serum to be fractionated to remove IgG antibodies, eliminate rheumatoid factor interference, and uses a specific anti-IgM conjugate. As with the standard western blot, there is no consensus as to the reactive bands required to qualify as a positive blot. Dobson *et al.* (1988) examined 5 symptomatic congenital syphilis cases and found reactivity with the 47kDa and 37kDa proteins. Similar asymptomatic cases were only reactive with the 47kDa band. The importance of the 47kDa protein has been further demonstrated by Sánchez *et al.* (1989) who fractionated sera from infants with congenital syphilis by high performance liquid chromatography. In all cases, they showed that the IgM reactivity was directed towards the 47kDa antigen. Several groups agree that the significant proteins include the 15.5, 17, 44.5 and 47kDa proteins, but other treponemal proteins also appear to be reactive (Lewis *et al.*, 1990; Sánchez *et al.*, 1989). Lewis *et al.* (1990) found IgM reactivity most frequently to the 47 and 37kDa proteins and less often to the 42, 34,5, 31 and 24kDa proteins. The criterion used was that there should be 5 distinct bands which must include the 47, 17 and 15kDa bands. They found 23 of 25 symptomatic infants with congenital syphilis gave positive blots using these criteria. The test was less sensitive in asymptomatic cases, but they concluded that it still offered superior sensitivity and specificity to the FTA-ABS 19S IgM test.

Polymerase chain reaction

The polymerase chain reaction (PCR) was invented in 1987 by Kary Mullis working in the Human Genetics Department at the Cetus Corporation (Mullis and Faloona, 1987). The discovery of *Taq* polymerase (Saiki *et al.*, 1988), a thermostable DNA polymerase isolated from *Thermus aquaticus*, turned PCR into a simple procedure. It is an *in vitro* technique for the enzymatic synthesis of specific DNA sequences using two synthetic oligonucleotide primers that hybridise to opposite

strands of a DNA template and flank the region of interest in the target DNA. All the components of the reaction are assembled in a single tube and subjected to a repetitive series of heating and cooling cycles. The reaction is heated to over 90°C to denature the template, then cooled to allow the primers to anneal, and then heated to 72°C to allow extension of the annealed primers by *Taq* polymerase. This thermal cycle is repeated many times. As the primer extension products from one cycle can act as a template in the next, the number of target DNA copies doubles at every cycle. This results in an exponential accumulation of the DNA fragment corresponding to the sequence between the two primers. Therefore, 20 cycles of PCR produces around a million-fold increase in the amount of target DNA present. The amplified DNA is usually analysed by agarose gel electrophoresis to identify if the diagnostic fragment is present.

PCR has been utilised effectively to aid in the diagnosis of a variety of infectious diseases, especially sexually transmitted diseases. It has had a significant impact on the diagnosis and management of HIV infection, and methods for the detection of *Chlamydia trachomatis* have become commercially available and FDA approved. The use of PCR in the field of syphilis is less well advanced. Most researchers have concentrated on its use in areas where conventional serological tests are ineffective, i.e. early infection, congenital syphilis, and neurosyphilis. They have tended to use sequences from within the lipoproteins of *T.pallidum* as targets for amplification because most had been cloned and their sequences were readily available in databases. Burstain *et al.* (1991) used as their target a 658 base pair segment of the *tpn*47 gene, which codes for the immunodominant TpN47 lipoprotein. They found that *T.pallidum* DNA could be amplified from paraffin-embedded tissue. The sensitivity of the test was determined using suspensions of organisms and was shown to be consistently positive with as few as 10 organisms.

The method was further evaluated for the diagnosis of congenital infection using samples of amniotic fluid, neonatal sera, and neonatal CSF (Grimprel *et al.*, 1991). The test detected all seven amniotic fluid specimens that were positive in the rabbit infectivity test, but was less sensitive with neonatal sera and neonatal CSF. It detected 3 out of 5 neonatal CSF, and 4 out of 6 neonatal sera that were positive by rabbit infectivity test.

The superior sensitivity with amniotic fluid may be because it is known to contain large numbers of treponemes. Hay *et al.* (1992) used primers derived from the sequences of two proteins, TpN44.5

(TmpA) and 4D. A positive result required both sequences to be amplified. They found a sensitivity of detection equivalent to 65 organisms in nonclinical specimens and tests using CSF specimens had an overall sensitivity of 58% in patients with a history of syphilis. However, 3% of patients with no known history of syphilis were also positive, which is a surprisingly high false positive rate. Noordhoek *et al.* (1991) used a nested PCR technique to amplify a 617bp fragment of the 39kDa basic membrane protein and showed the method to be extremely specific in an animal model (Wicher *et al.*, 1992). It was capable of detecting the isolated DNA from a single treponeme, but was less sensitive when used on CSF, detecting the equivalent of 100 organisms/mL. The method was evaluated using archived CSF specimens from neurosyphilis cases and detected 5 of 7 cases with symptomatic neurosyphilis and 2 of 16 patients with asymptomatic neurosyphilis. DNA could be detected in the CSF of patients long after intravenous penicillin treatment and so is probably of limited value in determining the efficacy of treatment.

This drawback highlights a major problem of using the presence of amplified DNA as a marker of infection. PCR will amplify the DNA from residual dead organisms present after successful treatment just as well as it does the DNA from live organisms causing active infection. This has been overcome in other infectious diseases by using a reverse transcriptase PCR (RT-PCR) method. RT-PCR uses RNA as the initial template from which a DNA copy is made, then a specific fragment is amplified as in a standard PCR. For amplification to occur disease specific RNA must be present and as RNA is quickly degraded live organisms must be present. Centurion-Lara *et al.* (1997) developed a RT-PCR method that targeted a 366bp region of the 16s rRNA of *T.pallidum*. It detected a single organism by Southern analysis when whole organisms were diluted, and 10^{-2} to 10^{-3} organisms when RNA equivalents were used to make cDNA. The test was demonstrated to detect 10^{-2} *T.pallidum* RNA equivalents in cerebrospinal fluid. They showed that 20 different isolates of *T.pallidum* from cerebrospinal fluid, aqueous humor, blood, and chancres, were detectable using the test. The test was shown to be more sensitive than a TpN47 DNA PCR method used for comparison. However, it needs to be evaluated on clinical specimens before it can be determined if it provides improved diagnosis of early disease, congenital infection, and neurosyphilis. It also remains to be seen if it provides new information on some of the anomalous results seen in treated neurosyphilis cases.

The use of PCR in early primary infection has been investigated using an animal model. *T.pallidum* DNA was amplified from whole blood, serous fluid and punch biopsies of skin lesions in experimentally infected rabbits (Wicher *et al.*, 1992). Of the 10 rabbits infected, nine were found to be positive by PCR eight days post infection. The rabbit that was negative by PCR failed to develop orchitis, suggesting the original infection failed. All specimen types were found to be equally effective, apart from serum where false negative results were obtained in all cases. These researchers thought that the treponemes may have been trapped during the clotting process. These results contradict the findings of others who reported reactive PCRs in 66% of neonatal serum specimens positive in the rabbit infectivity test (Grimprel *et al.*, 1991).

Generally, experimenters have found PCR to be highly specific and extremely sensitive, but prone to problems associated with specimen inhibition of the reaction and false positives due to laboratory contamination. PCR could be extremely valuable in diagnosing infection in situations were conventional serological methods have limitations, i.e. early primary syphilis, congenital infection and neurosyphilis. However, PCR remains confined to the research laboratory due to the specialised equipment required and uncertainties over the interpretation of results.

Serodiagnostic efficacy

The serological diagnosis of syphilis is traditionally based on identifying positive sera with a screening test, followed by retesting using a confirmatory method. This has the effect of increasing the predictive value of a positive result. In populations where the incidence of positives is low, even tests of high specificity have low positive predictive values. This is because the few false positive reactions that do occur are greater than the number of true positives detected. Using a second method to test the positives from the first has the effect of increasing the incidence of positives in the second test population. So, even if the second method has the same specificity as the first it will increase the predictive value of a positive result. Also, it is unlikely that a specimen will be falsely reactive in two different methods. However, the sensitivity of the confirmatory test must not be lower than the screening method or there would be a danger of false negatives by failing to confirm true positives. So, due to the low incidence of positives in the population, testing using a combination of methods is standard practice in syphilis serology.

Screening

There are three main screening strategies commonly employed, a cardiolipin antigen test or a treponemal test, such as the MHA-TP, used alone, or a combination screen using both together. Cardiolipin antigen tests continue to be used as screening tests because of their speed, simplicity and low costs. They all detect non-treponemal anti-cardiolipin antibodies that are released in response to the infection and become positive 7 to 10 days after the appearance of the primary chancre. The sensitivity ranges from 60% to 87% in primary infection and reaches 100% in secondary syphilis. There is a reduction in reactivity in the later stages of the disease, with about 30% of cases of late latent syphilis being non-reactive (Luger, 1988), and they become negative after successful treatment. Cardiolipin tests suffer from what is known as biological false positives, patients with a repeatedly reactive test but no history of syphilis, in about 0.3 to 0.9% of specimens. They are also prone to false negative results due to the prozone effect caused by high concentrations of antibodies. A prozone effect is thought to occur in about 1% of cases of secondary syphilis, although some reports suggest this is an underestimate of the problem (Spangler *et al.* 1964). To overcome this problem sera must be tested at a series of dilutions, which greatly increases the testing required. For these reasons, it is not advisable to test solely with a cardiolipin test, although in areas of high prevalence or limited resources it may still be considered a viable option.

Screening with a single haemagglutination test is effective for all stages of syphilis, except in early infection where the sensitivity is considered to be lower than that of a cardiolipin test. Haemagglutination assays are very sensitive and specific methods for the detection of *T.pallidum* antibodies. False reactions are rare, only 0.07% of specimens give a false positive result and 0.008% a false negative result (Luger, 1988). One long term study that looked at serodiagnosis over a 20 year period (Anderson *et al.*, 1989) found the sensitivity of cardiolipin tests to be 70% compared to 75% for haemagglutination tests in untreated primary syphilis. Another study (Young *et al.*, 1992) found the sensitivity of cardiolipin tests and haemagglutination to be 73% and 71%, respectively. So, on balance the two tests are fairly similar in performance in early infection. This has been taken by some authors to suggest that haemagglutination may be used as a sole screening method (Diggory, 1983). Generally, this is only considered viable for situations where early infections are not suspected.

The most common screening strategy employed is a combination of cardiolipin test and haemagglutination assay. When used together, these two tests provide a better screen than either of them used alone. Young *et al.* (1992) found the sensitivity of a combination of cardiolipin test and haemagglutination assay to be 84%, compared to 73% and 71% when used separately. This testing strategy offers excellent performance that cannot be surpassed, but it requires considerably more work than does a single test and it is not suited to automation.

Enzyme immunoassay is a method ideally suited to automation and the testing of large numbers of specimens. It is a sensitive and specific method capable of matching the performance of other treponemal tests and is suitable as a direct replacement for treponemal haemagglutination tests. The effectiveness of a method as a single screening test relies on its ability to detect early infections. Evaluation of a commercially available enzyme immunoassay (Captia® Syphilis-G) showed that it had a greater sensitivity in early infection than any other test, 82% compared to 76% for dark field microscopy, 73% for VDRL, 71% for TPHA, and 80% for FTA-ABS (Young *et al.*, 1992). A combination of VDRL and TPHA gave a slightly higher sensitivity of 84% that was not statistically significant. The authors concluded that the assay was a suitable replacement for the combination screening strategy. Considering the automation and direct data transfer advantages that enzyme immunoassay methods have to offer, they are an attractive proposition for laboratories requiring a high specimen throughput. Enzyme immunoassay may be used in either of two ways, as a single method screen, or as a replacement for a haemagglutination test in a combination screen. The excellent sensitivity and specificity shown by this test are why it was used for comparative purposes in the study reported here.

Confirmation

FTA-ABS is the most widely used confirmatory test. It has a reputation for being very sensitive and highly specific, but this reputation is a result of the dual testing strategy employed increasing the predictive value of a positive result. The specificity varies from 91% to 99% (Hunter *et al.*, 1986) and the sensitivity from 83% to 89% depending on whether borderline results are included or excluded (Larsen *et al.*, 1986). The prevalence of false reactions is around 1% in normal individuals, but may be much higher in hospitalised patients. This means that if the FTA-ABS test was used for screening it would give an unacceptable number of false reactions. The relatively poor specificity of FTA-ABS test has led some authors to suggest the use of enzyme immunoassay as

an alternative confirmatory test. Several commercially available enzyme immunoassays have been recommended as confirmatory tests (Lefevre *et al.*, 1990; Moyer *et al.*, 1987; Burdash *et al.*, 1987). The nature of enzyme immunoassay methods and the potential for automation makes them more suited to screening than confirmation. Western blotting has been investigated as an alternative confirmatory test (Dettori *et al.*, 1989; Byrne *et al.*, 1992). Most investigators find it a highly specific method, well suited to use as a confirmatory test. However, more work needs to be done to define the exact criteria that constitute a positive result. The identification of the diagnostically significant antigens of *T.pallidum* is crucial to this requirement.

Much of the discussion over the advantages and disadvantages of one testing strategy over another centres on their sensitivity in early infections. It must be remembered that all infectious diseases have a window between Infection and the development of a detectable antibody response. No testing strategy will detect all early infections, but as many as 15% of cases of primary syphilis were found to be serologically non reactive at presentation (Anderson *et al.*, 1989). There is clearly a need for tests with greater sensitivity than the existing strategies can deliver. The route most likely to succeed is through the use of recombinant protein to allow only the diagnostically significant antigens to be present in the test. This would have the effect of improving the specific activity of the antigen preparations thereby increasing test sensitivity. The crude antigens used in existing serological tests contain large amounts of non specific material and common antigens, which contribute to high number of false reactions suffered by all serological tests for syphilis. Recombinant proteins may help reduce the rate of false reactions by improving the specificity of antigens used. This is the starting point for the work reported in this study.

The organism

Taxonomy

The Treponema genus covers a variety of bacteria, some only very distantly related, whose shared classification is mainly based on their morphology and the periplasmic location of their flagella. They cause a range of diseases in humans and animals, including venereal syphilis, endemic syphilis, yaws, pinta, and spirochaetosis of rabbits. *Treponema pallidum* subsp. *pallidum* (venereal syphilis), *Treponema pallidum* subsp. *endemicum* (endemic syphilis), *Treponema pallidum* subsp. *pertenue* (yaws), *Treponema carateum* (pinta), and *Treponema paraluiscuniculi* (spirochaetosis of rabbits)

are virtually identical in terms of morphology, protein composition, and where tested, DNA homology (Fieldsteel, 1983; Maio and Fieldsteel, 1978, 1980; Maio *et al.*, 1978). They are classified as separate organisms on the basis of their different patterns of infection in humans and laboratory animals (Turner and Hollander, 1957). *Treponema carateum* and *Treponema paraluiscuniculi* are not classified as subspecies of *T.pallidum* due to lack of genetic information (Smibert, 1984). *Treponema pallidum* subsp. *pallidum, endemicum* and *pertenue* show greater than 95% DNA homology (Fieldsteel, 1983.; Maio and Fieldsteel, 1978, 1980), but less than 5% homology with *Treponema phagedenis*, *Treponema refringens* and *Serpulina hyodysenteriae* (Maio and Fieldsteel, 1978; Maio *et al*, 1978).

Pulsed-field gel electrophoresis studies have shown the *T.pallidum* genome to be a circular chromosome of approximately 1000 kbp, making it one of the smallest prokaryotic genomes (Saint Girons *et al.*, 1992; Walker *et al.*, 1991). Recently, the complete genome sequence was determined and shown to be 1,138,006 base pairs containing 1041 predicted coding sequences (Fraser *et al.*, 1998). Analysis of the sequence has shown systems for DNA replication, transcription, translation, and repair are intact, but catabolic and biosynthetic activities are minimised. The number of identifiable transporters is small, and no phosphoenolpyruvate: phosphotransferase carbohydrate transporters were found. Potential virulence factors identified included a family of 12 potential membrane proteins and several putative haemolysins.

Growth

Neither the human pathogenic treponemes or *Treponema paraluiscuniculi* are cultivable solely *in vitro*, but the other treponemal species can be quite easily cultured in liquid media under anaerobic or microaerobic conditions. *T.pallidum* is propagated by intratesticular infection of rabbits for research and diagnostic purposes. Fieldsteel *et al.* (1981, 1982) achieved limited *in vitro* culture, also confirmed in other laboratories (Norris and Edmondson, 1986, 1987), by co-cultivation with Sf1Ep cottontail rabbit epithelial cells in a special culture medium under microaerophilic conditions. They achieved approximately 100-fold increase in cell numbers, but subculture and long term *in vitro* growth were not successful. The fastidious nature and slow growth, both *in vivo* and *in vitro*, suggest the organism may have metabolic limitations or requirements for unidentified host products.

Morphology

T.pallidum is a corkscrew shaped organism with rigid, uniform, tightly wound, deep spirals. It is 6 to 20 µm long, 0.10 to 0.18 µm wide, with a spiral wavelength of 1.0 to 1.5 µm, and an amplitude of 0.5 to 0.7 µm. It is highly motile and moves with a characteristic motion, a deliberate backward and forward movement with rotation about the longitudinal axis. When attached to a heavier obstacle it bends and contorts, but springs back to shape on release. The basic structure resembles that of Gram-negative eubacteria in the presence of an outer membrane (Sell and Norris, 1983; Penn *et al.*, 1985). The outer membrane consists of a fragile lipid bilayer that is highly sensitive to detergent solubilisation and lacks lipopolysaccaride. It has been shown to have a low protein to lipid ratio and freeze-fracture studies show very few intramembranous particles compared to other similar bacteria (Walker *et al.*, 1991). The cytoplasmic membrane forms a complex with a thin peptidoglycan layer and, along with the outer membrane, encloses a periplasmic space. The periplasmic space contains structures known as endoflagella or periplasmic flagella. Between 2 and 4 flagella originate from each end of the cell and run along the longitudinal axis towards each other, overlapping in the middle. They are about 17nm wide and extend over more than half the length of the organism (3 to 10 µm). They are helical in shape and retain this shape when released from the cell, indicating the spiral shape of the organism is at least partly due to the flagella structure. The flagella consist of an inner core, approximately 12nm in diameter, surrounded by an outer protein sheath. Treponemes contain unique structures located within the cytoplasm, known as cytoplasmic filaments. These ribbon like structures, with no known function, are about 7nm wide and run the length of the organism just underneath the cytoplasmic membrane, parallel with the flagella (Masuda and Kawata, 1989).

Major polypeptides of T.pallidum.

The key to understanding the pathogenesis and physiology of *T.pallidum* is the identification of functional elements within the organism. As the organism cannot be cultured *in vitro,* only limited amounts of material have been available for study which has limited the type of experimental procedures that can be used. The purification of the structural components of *T.pallidum* has been an important step in the identification of proteins associated with the outer membrane, cytoplasmic membrane, flagella, and other structures. This has been particularly effective in characterising the structure and composition of the periplasmic flagella. The isolation of specific membrane

components has proved to be more problematic due to the highly labile nature of the outer membrane and the inability to differentiate outer membrane components from cytoplasmic ones. The advent of molecular biology techniques has allowed a large number of treponemal proteins to be cloned and expressed in *E.coli.* A complete list of all cloned proteins and plasmid constructs has been compiled by the *Treponema pallidum* Polypeptide Research Group (Norris *et al.*, 1993). However, their function and phenotypic effect have been difficult to determine because deletion mutants can not be constructed due to the inability to culture the organism *in vitro.* Sequence homology with proteins of known function from other bacteria has been used to ascribe a putative function to some treponemal proteins. So, although a large number of *T.pallidum* proteins have been identified, their structural significance and functions remain unknown in many cases. Generally, these proteins were isolated from cDNA libraries by an immunoscreening method using hyperimmunised anti-*T.pallidum* antiserum, therefore they are antigenic by definition. These proteins represent a range of potentially diagnostically significant antigens of *T.pallidum* for evaluation and consideration.

Nomenclature

At one time different laboratories had their own designations for *T.pallidum* polypeptides, which made exchange of data and comparisons between laboratories difficult. Norris *et al.* (1987) compared SDS-PAGE patterns of *T.pallidum* from different laboratories and showed this method to be inadequate to definitively identify the polypeptides. A method using 2DGE in combination with monoclonal antibodies yielded adequate differentiation and was used as the basis of the current nomenclature (Norris *et al.* 1993). The system consists of the prefix TpN followed by the relative molecular mass of the protein based on consensus SDS-PAGE results. The corresponding gene is indicated by lower case italics. So, the 17kDa protein, TpN17 is coded for by the gene designated *tpn17.*

Flagellar proteins

The periplasmic flagellum is the best characterised structure of *T.pallidum* (Charon *et al.*, 1992), probably because they are readily isolated by differential centrifugation. The flagella of *T.pallidum* consist of multiple proteins arranged into an outer sheath and a central core (Penn *et al.*, 1985; Radolf *et al.*, 1986; Cockayne *et al.*, 1987; Blanco *et al.*, 1988). This contrasts with other bacteria where the flagella are constructed of multiple repeats of a single polypeptide subunit (Namba *et al.*,

1989). The flagellar proteins are highly immunogenic which has facilitated their identification and characterisation by electrophoretic and immunoblotting techniques. Six flagella-associated proteins have been identified, cloned, and sequenced, TpN37a, TpN34.5, TpN33, TpN30, TpN29, and TpN27.5. TpN37a has been designated FlaA, and TpN34.5, TpN33, and TpN30 have been designated FlaB1, FlaB2, and FlaB3, respectively, on the basis of DNA sequences and antigenic similarities.

FlaA

The *FlaA* gene was cloned and sequenced (Isaacs *et al.*, 1989), and found to code for a protein of 37kDa. The promoter region contains -35 and -10 sequences that show consensus with those sequences known to be recognised by σ^{70} transcriptional factors (Isaacs *et al.*, 1990). The mature protein sequence is preceded by a hydrophobic signal peptide and a consensus signal peptidase I cleavage sequence. This suggests that the signal peptide is cleaved off the protein translation product and FlaA is transported across the cytoplasmic membrane. FlaA appears only to be present in certain spirochaetes and is highly conserved in the sequences studied (Parales and Greenberg, 1991, 1993). It shows no sequence homology with conventional bacterial flagellins. FlaA is thought to be secreted into the periplasmic space and overlaid on to the growing flagellar core to form the outer sheath of the flagella. This association with the outer sheath has been identified using monoclonal antibodies which lose reactivity after removal of the outer sheath of the flagella (Cockayne *et al.*, 1987).

FlaB

FlaB1 and *FlaB3* were cloned as a single DNA fragment and are closely associated (Champion *et al.*, 1990). *FlaB3* is only 281 base pairs down stream of *FlaB1* and lacks a promoter sequence. *FlaB1* is preceded by a consensus σ^{28} promoter sequence, so it is possible that the two genes are expressed as a polycistronic mRNA. *FlaB2* was cloned and sequenced separately and was found to be expressed from a separate consensus σ^{28} promoter sequence (Pallesen and Hindersson, 1989). FlaB1, FlaB2, and FlaB3 are similar sized proteins and exhibit a high degree of homology with each other. The FlaB proteins show a high degree of homology to flagellins of other bacteria, particularly in the N-terminal region. As described with other bacteria (Namba *et al.*, 1989), the FlaB proteins are thought to be secreted with an intact N-terminal sequence and transported through the flagellar

base unit and core to be added to the end of the flagella. However, the exact relationship between the individual proteins is unknown.

Other flagellins

TpN29 and TpN27 are two proteins of unknown structural or functional significance which are detected in some flagellar preparations (Norris *et al.*, 1988). They have not been cloned and do not appear to be degradation products of other flagellins because monoclonal antibodies directed against FlaA and FlaB proteins do not react with them. The recent availability of the full genome sequence may facilitate the cloning of these proteins and allow putative functions to be assigned.

Membrane associated proteins

The ability of *T.pallidum* to evade the immune system, and the fastidious nature of its growth requirements, suggest that the membrane structure may be of importance due to its metabolic involvement and immunogenic properties. The membrane associated proteins that have been isolated represent prime candidates for diagnostically significant antigens. The surface of *T.pallidum* has long been thought to be relatively inert, behaving as if it lacked proteins and antigens (Penn *et al.*, 1985). The application of conventional cell fractionation techniques that had been used successfully on other Gram-negative organisms, failed to produce significant information. As a result phase partitioning experiments with the anionic detergent, Triton X-114 (Bordier, 1981), were employed. The solubilisation of the membrane recovered nearly all the major antigens of *T.pallidum* in the detergent enriched fraction (Radolf and Norgard, 1988; Cunningham *et al.*, 1988), indicating their hydrophobic nature. A large number of these proteins were shown to be lipidated and strongly antigenic in humans and animals. So far, eight major antigenic lipoproteins of *T.pallidum* have been cloned, sequenced, and characterised.

The strong antigenicity of these lipoproteins was in contradiction to the inertness of the outer membrane. This suggested that these lipoproteins were not surface exposed and this inference was further reinforced by freeze-fracture electronmicrography studies. These studies showed that the fracture faces contained approximately 100-fold fewer intramembranous particles (IMP) than other similar organisms (Walker *et al.*, 1989, 1991). These IMPs were designated treponemal rare outer membrane proteins, or TROMPS.

The exact location of the major lipoproteins of *T.pallidum* was difficult to establish due to the intrinsic instability of the outer membrane resulting in periplasmic associated proteins becoming surface exposed. The major immunodominant antigen, TpN47, was located to the cytoplasmic membrane using immunoelectron microscopy of ultra-thin cryosections (Cox *et al.*, 1992). Using a novel immunofluorescence technique in which treponemes are encapsulated in porous gel beads, four of the major antigens, TpN47, TpN34, TpN17 and TpN15, were shown to be associated with the cytoplasmic membrane (Cox *et al.*, 1995). A method was developed without the use of detergents that produced a pure outer membrane preparation (Blanco *et al.*, 1994). This was shown by the absence of the abundant lipoprotein TpN47 and the previously characterised cytoplasmic 4D protein (see below) from isolates prepared by the method. Analysis of these outer membrane fractions showed the presence of six protein species of 17kDa, 28 kDa, 45 kDa, 65 kDa, and two 31kDa proteins with distinct isoelectric points. The proteins of 17kDa and 45kDa were shown to be the lipoproteins, TpN17 and TpN44.5, by their physical properties and reactivity with specific monoclonal antibodies. Over 90% of these lipoproteins remained associated with the cytoplasmic membrane but their presence also in the outer membrane fraction was not thought to be contamination in view of the absence of the normally abundant TpN47. The remaining 31kDa, 28kDa, and 65kDa proteins were considered true outer membrane proteins and designated Tromp1, Tromp2 and Tromp3, respectively.

Lipoproteins

Using phase partitioning with detergents to study the *T.pallidum* membrane structure resulted in a large number of proteins being identified. Many of these proteins were found to be lipidated and highly immunogenic. Eight major lipoproteins have been identified and represent candidates for evaluation as diagnostically significant antigens. They have been cloned by several groups from genomic libraries and sequenced. Table 1 summarises the lipoproteins and the groups responsible for their identification and characterisation.

Table 1. Lipoproteins of *T.pallidum*

Protein designation	M_r	Deduced MW	N-terminal sequence	References
TpN47 (47K)	47,000	46,740	MKVKYALLSAGALQLLVVG ↓ CG	Chamberlain *et al.*, 1989; Hsu *et al.*, 1989; Weigel *et al.*, 1992
TpN44.5 (TmpA)	42,000	42,000	MNAHTLVYSGVALACAAMLGS ↓ CA	Hansen *et al.*, 1985; Schouls *et al.*, 1989; Yelton *et al.*, 1991
TpN39 (38K)	38,000	36,194	MKENSCTACSRRLALFVGAAVLVVG ↓ CS	Bailey *et al.*, 1989
TpN35 (TmpC)	35,500	35,600	VREKWVRAFAAVFCAMLLIG ↓ CS	Hubbard *et al.*, 1991; Schouls *et al.*, 1991
TpN29-35 (34K, TpD)	30,000 - 38,000	22,087	MKRVSLLGSAAIFALVFSA ↓ CG	Schouls *et al.*, 1989; Swancutt *et al.*, 1986, 1989, and 1990
TpN24-28 (TpE)	24,000 - 30,000	ND		Bailey *et al.*, 1989; van Embden *et al.*, 1983
TpN17 (Tpp17)	17,000	14,438	MKGSVRALCAFLGVGALGSAL ↓ CV	Akins *et al.*, 1993; Chamberlain *et al.*, 1989
TpN15 (Tpp15)	15,000	13,967	MVKRGRFALCLAVLLGA ↓ CS	Purcell *et al.*, 1989, and 1990

Alternative designations are shown in brackets. The relative molecular weights are based on SDS-PAGE. The deduced molecular weights are for the mature polypeptides, without lipid attachments, based on DNA sequence data. The table is modified from Norris *et al.* (1993).

All the lipoproteins have a hydrophobic signal sequence of between 17 and 21 amino acids followed by a consensus signal peptidase II recognition site. The consensus recognition sites are a leucine, valine or serine residue separated from a cysteine by 2 or 3 hydrophobic amino acids. The signal sequence is bound by the enzyme, the lipid added to the cysteine residue through an amide linkage and the formation of a diacylglyceryl thioester bond, and then the signal sequence is cleaved (de Vrije *et al.*, 1990). The importance of the cysteine residue has been shown by mutation to a serine residue resulting in non-lipidated protein being produced (Schouls *et al.*, 1991). The sequences, apart from the signal sequence, are hydrophilic which suggests that the partitioning to the detergent phase is due to their lipid content and not the amino acid composition. The lipoproteins are highly immunogenic and thought to be located in association with the cytoplasmic membrane. None of

these proteins show significant homology with other bacterial or eukaryotic sequences in databases, and so have no putative functions assigned to them.

Other membrane associated proteins

TpN39b (basic membrane protein)

This positively charged, hydrophobic protein was cloned and sequenced by Dallas *et al.* (1987). The deduced N-terminal amino acid sequence contains a signal peptide and signal peptidase I cleavage site.

TpN34 (TmpB)

TmpB was cloned along with the lipoprotein TmpA (TpN44.5) by Hansen *et al.* (1985). The open reading frame of TmpB overlaps the 3' end of the TmpA encoding region by one base pair. The two proteins are expressed as a polycistronic mRNA from a single RNA transcriptional initiation site.

Treponemal rare outer membrane proteins

Tromp1

The amino acid sequence of the 31kDa protein from outer membrane fractions was determined and used to clone the encoding structural gene, which was designated *tromp1* (Blanco *et al.*, 1995). The recombinant protein was used to generate antisera which showed both proteins originally identified were Tromp1. The N-terminal region of the protein consists of a hydrophobic sequence and a putative signal peptidase I cleavage site. The protein was predicted to contain 14 membrane spanning beta pleated sheet segments, highly analogous to Gram-negative outer membrane proteins. Native Tromp1 was demonstrated to have porin activity in the black lipid bilayer assay which suggests it may be an outer-membrane spanning protein. The controlled expression of recombinant Tromp1 in *E.coli* showed it to be localised to the outer membrane fraction and porin activity of recombinant Tromp1 was demonstrated using the black lipid bilayer assay (Blanco *et al.*, 1996). The work of Blanco and colleagues suggests that Tromp1 is a surface-exposed protein with porin like properties. However, no evidence for either outer membrane location or surface exposure of native Tromp1 within motile or intact treponemes has been presented. The *tromp1* gene has been found to be part of an ABC transport operon with significant sequence homology to a family of Gram-negative periplasmic substrate-binding proteins (Hardham *et al.*, 1997).

The apparent discrepancies between the studies of Blanco and colleagues and this sequence data prompted work to resolve the differences. Akins *et al.* (1997) showed that Tromp1 was probably a substrate-binding protein anchored to the cytoplasmic membrane by an uncleaved signal sequence. They could not show porin activity and suggested the previous reactivity seen in the black lipid bilayer assay may have been due to the presence of detergent or other contaminants. The differences in the predicted secondary structure were attributed to atypical processing events due to the recombinant Tromp1 being fused to the *E.coli* OmpT leader sequence. Tromp1 produced in an *in vitro*-coupled transcription-translation reaction, lacking signal peptidase I activity, resulted in a protein of the same size as native Tromp1. This is inconsistent with cleavage of the N-terminal signal sequence. However, recently Blanco *et al.* (1999) have shown, using mass spectrometry, that purified native Tromp1 is processed and does not contain an uncleaved signal sequence as previously reported (Akins *et al.*, 1997). So, true outer membrane proteins remain to be conclusively identified.

Tromp2

Amino acid sequences were used to amplify and clone the structural gene coding for the 28kDa outer membrane protein, now designated Tromp2 (Champion *et al.*, 1997). The deduced amino acid sequence showed a 24 amino acid N-terminal hydrophobic signal sequence and a signal peptidase I cleavage site. Protein modeling suggests a structure of 9 membrane spanning beta pleated sheet segments. In contrast to Tromp1, Tromp2 does not seem to be toxic to *E.coli* and even maximum expression levels result in the protein being localised to the outer membrane. Evaluation in the black lipid bilayer assay shows only occasional channel formation even at high levels of membrane insertion. Thus, whether Tromp2 has porin activity is not clear.

Tromp3

The 65kDa protein identified in outer membrane preparations has been tentatively designated Tromp3. It occurs in smaller, more variable amounts than the other outer membrane proteins which suggests that it may be differentially expressed. The small quantities of protein available have prevented amino acid sequencing and cloning.

Other proteins

TpN19 (4D)

The 4D antigen was one of the first *T.pallidum* proteins to be cloned in *E.coli* (van Embden *et al.*, 1983). The native molecule has a molecular weight of around 190kDa and is composed of ten 19kDa subunits which form a ring-like structure about 6nm in diameter (Fehniger *et al.*, 1986). It can be purified as a separate entity on sucrose density gradients and is thought to be located within the cytoplasm. The function of the 4D antigen is unknown and no homologous sequences have been identified.

TpN60 (GroEL)

TpN60 is a polypeptide of approximately 60kDa and a major component of T.pallidum. The native molecule is a ring-like structure with a molecular weight of around 800,000. It was shown to immunologically crossreact with similar proteins from other bacteria (Hindersson *et al.*, 1984). Cloning (Hindersson *et al.*, 1987) and sequence analysis show that it is homologous to the GroEL heat shock protein of *E.coli* (Houston *et al.*, 1990).

Antibody responses in syphilis

The study of the proteins of *T.pallidum* by conventional purification and functional analysis techniques has been hampered by the inability to culture the organism *in vitro*. So, the focus of many research groups has been to examine and catalogue the important proteins and polypeptides using antibodies from immunised animals and human syphilitic patients. As a result, the antibody response during syphilis has been extensively investigated by many groups of researchers using a variety of approaches, including crossed immunoelectrophoresis, SDS-polyacrylamide gel electrophoresis and western blotting, radioimmunoprecipitation, and two dimensional electrophoresis. A large body of information exists which is difficult to interpret due to the inconsistent identification of proteins between groups. This may be due to differences in the methods used to separate the organisms from rabbit tissue, differences due to the electrophoresis format used, and different or ill-defined molecular weight markers. Although the SDS-PAGE patterns of *T.pallidum* reported by different laboratories were shown to be quite similar (Penn *et al.*, 1986), considerable uncertainty existed with regard to the identity and molecular weights of polypeptides described by different researchers. A collaborative study compared the results of different groups, their methods, and used monoclonal antibodies to identify and define various

proteins of *T.pallidum* (Norris and Edmondson, 1987). A set of consensus molecular weights for the proteins of *T.pallidum* were obtained, which formed the basis for the nomenclature system described by the *Treponema pallidum* Polypeptide Research Group (Norris *et al.*, 1993). This provides a means of reinterpreting existing information from participating laboratories in relation to current nomenclature, where suitable molecular weights were assigned. However, a large number of papers remain impossible to interpret due to the lack of established standards or a means of comparing the results to those of the participating laboratories. The experimental work described in the results section of this thesis also plays an important role in providing an identity to the proteins cited in the studies discussed below.

The antibody response has been studied using two strategies, experimental infection of laboratory animals, and the analysis of serum specimens from patients with different stages of syphilis infection. The experimental infection of rabbits allows the progress of the infection to be monitored much more closely than is possible using human serum specimens. Hanff *et al.* (1983) experimentally infected rabbits and monitored the appearance of reactivity to the various proteins over several months. By the 9th day post infection, they found weak reactivity to proteins that were probably TpN60, the GroEL homologue or common antigen, TpN47, and several flagellar proteins, TpN37, TpN33 and TpN30. The next proteins to become reactive were two low molecular weight species, probably TpN17 and TpN15. As the infection progressed so the reactivity of all proteins increased proportionately until by 1 month post infection there was reactivity to more than 21 distinct polypeptides. Lukehart *et al.* (1986) conducted a similar study, but also measured the IgM as well as the IgG responses. The initial IgM responses were to proteins which were probably TpN47, TpN44.5, and TpN37, followed by reactivity to TpN17 and TpN15 a few days later. The IgG response developed after the IgM response and was initially directed towards TpN37, followed by reactivity to TpN47.

Although not in full agreement, these studies show broadly similar findings which have also been demonstrated by others (Alderete and Baseman, 1981; Wicher *et al.*, 1991). The course of syphilis in man has been studied using serum specimens from patients at different stages of syphilis, so accurate time course experiments are impossible. The information is restricted to the broad categories into which the disease can be clinically subdivided, early primary infection, secondary or

early latent syphilis, latent syphilis, and the later destructive stages like neurosyphilis or cardiovascular syphilis.

Van Eijk and van Embden (1982) found different immunoglobulin subclasses to be present at different stages of the disease. In early primary infection there are IgM, IgG, and IgA responses to a protein, probably TpN47, and IgM only to what was probably TpN17, with IgA to probable TpN15. In secondary syphilis the IgA response disappeared and the IgM reactivity was the same as in early infection but with the addition of reactivity towards TpN37. IgG reactivity was seen to TpN47, TpN44.5, TpN37, TpN17, and TpN15. In latent syphilis and neurosyphilis they found only IgG reactivity, with the same reaction pattern as in secondary syphilis. This study identified surprisingly few proteins compared to other studies, particularly the IgG responses in secondary syphilis where one might expect upwards of 20 distinct polypeptides to be identified.

Hanff *et al.* (1982) showed that serum IgG from uninfected individuals reacted weakly with three polypeptides, probably TpN47, TpN33 and TpN30. They found IgM antibodies in patients with primary infection and IgG antibodies in all patients with syphilis. In early primary infections the reactivity was to proteins that were probably TpN47, TpN33, TpN30 and TpN15. Antibody to TpN44.5 and TpN17 were markers for non primary infection. In secondary and early latent syphilis reactivity to a large number of protein bands were identified which reduced in number and intensity as the disease progressed to the later stages. In the later stages of the disease, strong reactivity remained to TpN47, TpN44.5, TpN17, and TpN15.

Another comprehensive study by Baker-Zander *et al.* (1985) showed similar results, except that they found IgM reactivity in secondary and early latent cases. Sera from early primary infections varied in reaction pattern, but were consistently strongly reactive with TpN47 and sera from secondary and early latent syphilis were uniformly reactive with 22 separate polypeptide antigens. Similar reactivities have been demonstrated by other researchers using radioimmunoprecipitation rather than western blotting (Moskophidis and Muller, 1984).

The work discussed above is by no means a complete review of all the available literature. The studies are some of the more comprehensive investigations of the antibody response where the proteins can be identified with some confidence, although a certain amount of intuition is still required. There are many other studies that simply confirm these findings and others that remain

uninterpretable in terms of current nomenclature. The rabbit infection experiments show a clearer consensus of the profile of immunogenic proteins and the time course of the appearance of detectable antibodies of different classes than do the human studies. This is probably because the patient specimens used tend to be a diverse population, categorized on clinical judgment, which leads to a more variable picture. However, although there is not complete agreement on the proteins antigenic at each stage of the disease, there are general findings from which conclusions can be drawn. The earliest responses, both IgG and IgM, are to TpN47 and the flagellar proteins. This is followed by the development of significant IgG reactivity to TpN44.5, TpN17 and TpN15. By the time secondary syphilis develops there are antibody responses to a large number of proteins in addition to those recognised in the earlier stages. The number and intensity of the responses decrease in latent and the later destructive stages of the disease, but reactivity to TpN47, TpN44.5, TpN17, TpN15, and several flagellar proteins remains. Several studies have shown reactivity to some of the flagellar proteins and TpN47 in the serum of a few normal individuals with no history of syphilis (Hanff *et al.*, 1982; Baker-Zander *et al.*, 1985). This is of potential concern as TpN47 is widely considered a pathogen-specific immunodominant antigen and important for the development of immunodiagnostic tests.

Sera from the experimental infection of rabbits, and human sera from infected individuals have been used to examine the cross reactivity between *T.pallidum* subsp. *pallidum* and other treponemes. Much effort has been expended in trying to distinguish between the different pathogenic treponemes for diagnostic and epidemiological purposes. The antibody response to infection with *T.pallidum* subsp *pertenue*, the causative agent of yaws, is indistinguishable from that seen in syphilis (Baker-Zander and Lukehart, 1983; Thornburg and Baseman, 1983). Fohn *et al.* (1988) found patients suffering from pinta, caused by *T.carateum*, also showed the same antibody reaction pattern as those with syphilis. The cross reactivity with non-pathogenic treponemes has long been of interest because of their cultivable nature and they have been used as sources of antigens for serological tests. *T.phagedenis* biotype Reiter, *T.refringens* strain Noguchi, and *T.vincentii* have all been shown to contain numerous proteins that cross react with antibodies to the proteins of *T.pallidum* subsp. *pallidum*, which indicates the conserved antigenic determinants of treponemes (Lukehart *et al.*, 1982; Wicher *et al.*, 1986). Lukehart *et al.* (1982) identified three pathogen specific proteins of *T.pallidum* using syphilitic rabbit serum that had been extensively

adsorbed with Reiter treponemes. The proteins identified were 48kDa, 14kDa, and 12kDa, which probably correspond to TpN47, TpN17 and TpN15.

In conclusion, the antigens most likely to be diagnostically significant are those proteins most frequently cited, TpN47, TpN44.5, TpN17 and TpN15. These proteins have all been cloned and sequenced by several groups of researchers, but few have been extensively evaluated as diagnostic reagents.

Diagnostic recombinant proteins

Established treponemal serological tests for syphilis are mainly based on antigens derived from sonicated *T.pallidum.* The cloning of *T.pallidum* proteins allowed them to be expressed and purified in large quantities. An obvious application of the technology was as a source of specific antigens to replace *in vivo* cultivated organisms in serological tests. Considering how many *T.pallidum* proteins have been cloned, very few have been evaluated as diagnostic antigens. TpN35 (Schouls *et al.*, 1991), or TmpC, and TpN29-35 (Hindersson *et al.*, 1986), or TpD, have been evaluated to a limited extent, making it impossible to assess their importance as diagnostic antigens. Three proteins that have been evaluated extensively are TpN19 (4D antigen), TpN38, a putative fibronectin-binding adhesin, and TpN44.5, a membrane associated lipoprotein.

Recombinant TpN19, previously called the 4D antigen, was evaluated by indirect enzyme immunoassay using an anti-IgG conjugate (Radolf *et al.*, 1986). Using stored specimens, the antigen was found to be negative with 172 VDRL negative sera and 20 specimens from patients with a biological false positive response. The sensitivities were 81% in early primary syphilis, 100% in secondary/early latent disease, but dropped to 57% in cases of neurosyphilis and cardiovascular syphilis. Using fresh sera from dark field positive patients with untreated primary infection the sensitivity was 83%, compared to FTA-ABS and VDRL which were both slightly better at 88%. Just like all other serological tests for syphilis, sera from patients with yaws and pinta were all positive.

Recombinant TpN38 was evaluated using a radioimmunoassay (Rogers *et al.*, 1986). It detected 71 out of 81 untreated and treated cases which were not defined by disease stage. This translated to a sensitivity of 87.7% and a specificity of 95.4% which compared unfavourably with FTA-ABS, which detected all the positives. The sensitivity was examined further using 50 untreated primary cases. Only 38 were positive, giving a sensitivity of 76% compared to FTA-ABS which gave 86%. Of the 42

biological false positives tested four were falsely positive, compared to none by FTA-ABS. In summary, this antigen appears to lack sensitivity compared to established tests and shows false reactions.

TpN44.5 (TmpA) and TpN36 (TmpB) were cloned together and found to be expressed from overlapping open reading frames. They were both evaluated as diagnostic antigens by Schouls *et al.* (1989) using an enzyme immunoassay format. TpN36 was found to be non reactive with a high proportion of the specimens tested and so was considered unsuitable for use as a single diagnostic antigen. TpN44.5 was evaluated much more extensively. It detected 21 out of 24 untreated primary syphilis cases giving a sensitivity of 87.5%. All the specimens that were positive by FTA-ABS were also positive in the recombinant assay. It was positive with all secondary (25) and latent (19) specimens tested, which were also all positive by VDRL and FTA-ABS. It was tested against specimens from patients with treated syphilis, 30 VDRL positive cases and 30 VDRL negative cases, and found to detect 25 and 10, respectively. In a second study (Ijsselmuiden *et al.*, 1989a) the assay detected 42 out of 55, or 76%, of untreated primary infections. Again, reactivity was almost universal in secondary and early latent cases, detecting 100% and 98% of cases, respectively. The lower sensitivity in this study in early infections was probably due to the inclusion of a number of specimens from dark field positive, FTA-ABS negative cases. Overall, the use of TpN44.5 as a single antigen produced results fairly similar to that expected from established treponemal tests, perhaps showing slightly less sensitivity in early primary infections. This test was licensed to Eurodiagnostics (Apeldoorn, Netherlands), but never became commercially available nor was its performance reported in peer reviewed journals.

TpN47 has been thought to be the immunodominant antigen of *T.pallidum* and reactivity to it features prominently in the immune response to syphilis. So, it is puzzling as to why the literature contains so few publications of the use of TpN47 as an antigen in an immunoassay, and none by the group that originally cloned the protein. The use of TpN47 for the study, diagnosis and production of vaccines for syphilis was covered in a patent assigned to the University of Texas (WO 8802403). As it is such a key protein in the immune response the patent rights were probably licensed for development of a commercial test. ADI Diagnostics (Ontario, Canada) produced an enzyme immunoassay which used non-acylated TpN47 as the solid-phase antigen which had little impact in the marketplace and there seems to be a lack of peer reviewed articles describing its

performance. The company's own product literature shows the sensitivity to be slightly lower in all stages of the disease than MHA-TP. This alone would probably mean the test would be of limited appeal to diagnostic laboratories.

Generally, the most surprising aspect is that although many proteins of *T.pallidum* have been cloned and the sequences are readily available in databases, very few have been evaluated as potential diagnostic antigens. From the work described above it can be seen that the use of single antigenic proteins has failed to yield the sensitivity required to produce superior performance to existing testing strategies. A combination of several antigenic proteins may be required to detect sufficient antibody specificities to provide adequate serodiagnosis at all stages of the disease, and hence attain the sensitivity required to replace existing tests.

Research objectives

Syphilis is a multi-stage disease with complicated antibody responses, where serological tests have to be effective over a wide range of disease presentations. The diagnostic tests currently in common use have limitations in terms of both sensitivity and specificity, which manifests as poor sensitivity in early primary infections and a high rate of false positive and negative reactions. This can mainly be attributed to the crude antigen preparations used in all existing serodiagnostic tests. There is an obvious requirement for more specific and sensitive serodiagnostic tests. There are also particular stages of the disease or complications where the deficiencies of the existing serological tests are more profound. In cases of HIV co-infection the antibody response is often undetectable by existing tests, and in congenital syphilis maternal antibody interferes, making the detection of foetal IgM an important diagnostic tool. These diagnostic applications are not supported by existing nontreponemal tests and haemagglutination assays. The diagnosis of neurosyphilis requires the detection of the organism in CSF, which is not easily achieved by microscopy, but is an application ideally suited to the use of PCR. Syphilis diagnosis still relies heavily on microscopy techniques and serological methods based on crude antigen preparations. The application of the techniques of modern molecular biology offers the opportunity to improve the accuracy of syphilis diagnosis. The use of recombinant proteins and enzyme immunoassay technology would improve the specificity and sensitivity of serological methods, immunoblotting could make confirmatory

testing truly specific, and PCR technology could successfully detect the presence of live organisms in body fluids.

However, this study is solely concerned with improved serodiagnostic methods. The most obvious route to improved serological methods is through the use of recombinant proteins as antigens. This is a strategy that has had a major impact on sensitivity and specificity of serodiagnostic procedures in many areas of infectious disease diagnosis. Considering the difficulties and expense of producing antigens in animals, it is surprising that recombinant protein antigens have not been utilised previously. Many of the proteins of *T.pallidum* have been cloned and sequenced. Generally, these proteins were isolated from cDNA libraries using immunoscreening methods, so most of them are antigenic to a greater or lesser extent. These represent a range of potential antigens for use in serological tests. However, the exact antigens that are diagnostically significant in syphilis is an area of controversy. Very few of the documented proteins have been evaluated as diagnostic reagents, and at the beginning of this project no tests using recombinant proteins were commercially available.

The initial objectives of this study were to confirm the diagnostically significant antigens of *T.pallidum*, by a combination of assessment of the literature and experimental work. Once identified, these antigens would become the targets for a series of experiments designed to clone, express and purify the recombinant proteins in order to provide material for evaluation. The antigenicity of each protein would be examined using sensitive immunoblotting techniques before being used for the development of an enzyme immunoassay method.

Although recombinant proteins are easier to produce and purer than native antigen, they still suffer some problems. Some recombinant proteins are difficult to express and purify, resulting in contamination with residual *E.coli* proteins. This can cause specificity problems due to crossreaction with antibodies to *E.coli* protein naturally occurring in the patient's serum. These problems are magnified when the immunoassay requires multiple recombinant proteins to express the antigenicity required for adequate diagnosis. These problems can be overcome by control of the purification scheme, but another solution is the use of synthetic peptide antigens which, due to the manufacturing process, offer the advantage of high levels of purity and are relatively inexpensive. In order to design suitable peptides it is essential to identify the sequences within the antigens responsible for their antigenicity. This is the subject of the second phase of the study reported here.

The objective was to identify the epitopes within the protein to which the antibody response was directed, by using a series of overlapping peptides complementary to the protein sequence. This phase would be supported using a complementary technique, known as phage display, to gain additional information on the types of epitopes present within the antigens. If short antigenically-significant sequences could be identified, then synthetic peptides could be constructed to express the antigenicity. If multiple sequences were identified these could be joined to make a compound synthetic peptide antigen for use in serological tests. An alternative possibility would be to express the antigenic sequences as an artificial recombinant protein.

To summarise the key objectives:

- Identify the diagnostically significant antigens of *T.pallidum*.
- Produce recombinant versions of these antigens.
- Test the antigenicity of the recombinant antigens in the different stages of syphilis.
- Use the recombinant antigens to develop an enzyme immunoassay.
- Identify the sequences within each protein responsible from their antigenicity.

Chapter 2

Materials and Methods

General DNA manipulation methods

Culture media

LB broth	**Amount/litre**
Tryptone	10g
Yeast extract	5g
NaCl	10g

Sterilise by autoclaving. LB agar was prepared by adding 1.5% (w/v) agar before autoclaving. LB top agar was prepared by adding 0.7% (w/v) agar before autoclaving.

NZY broth	**Amount/litre**
Casein hydrolysate	10g
Yeast extract	5g
NaCl	5g
$MgSO_4.7H_2O$	2g

Sterilise by autoclaving. NZY agar was prepared by adding 1.5% (w/v) agar before autoclaving. NZY top agar was prepared by adding 0.7% (w/v) agar before autoclaving.

x10 M9 salts	**Amount/litre**
$Na_2HPO_4.7H_2O$	128g
KH_2PO_4	30g
NaCl	5g
NH_4Cl	10g

Dissolve in water to a final volume of 1 litre and sterilise by autoclaving.

M9/+Thl minimal medium	**Amount/litre**

16.8g of agarose was added to 1L of water and autoclaved. It was cooled to 50°C and the following components added, mixed and dispensed into petri dishes.

x10 M9 salts	100mL
20% glucose*	20mL
1M $MgSO_4$**	2mL
1M $CaCl_2$**	0.1mL
10mg/mL thiamine*	1mL

* 0.2µm filter sterilise
** autoclave separately

*Bacterial strains (*E.coli*)*

XL-1blue

*recA*1, *endA*1, *gyrA*96 *thi*-1, *hsdR*17, *supE*44, *relA1*, *lac*-, F'[*proAB, lacI*^{q}ZΔM15 Tn*10* (tet^r)]

XL-1blue cells carry the F' episome encoding pili that allows infection by filamentous M13 phage or phagemid vectors. The F' episome can be maintained by growth in the presence of tetracycline. It contains the *lacI*^{q}ZΔM15 mutation which provides α-complementation of the β-galactosidase gene. This allows blue/white colour selection of recombinant colonies when supplemented with X-gal and Isopropyl-β-D-thiogalactopyranoside (IPTG). The F' episome encoded *lac*^{q} produces 10-fold more *lac* repressor than is found in most host strains. This ensures stringent repression of any effects from cloned inserts. XL-1blue cells are restriction negative (hsdR17), recombination (recA1) and

endonuclease deficient (endA1). These mutations prevent the cleavage of cloned DNA, enhance insert stability and improve plasmid minipreps, respectively. XL-1blue was used for all routine cloning experiments. The strain was maintained by growth on LB agar containing 12.5µg/mL tetracycline.

TG1

supE, *hsd*Δ5, *thi*, Δ(*lac-proAB*), F'[*traD*36, *proAB*+ *lacI*^{q} *lacZ*ΔM15]

TG1 cells carry the F' episome which allows infection by filamentous M13 phage and phagemids. The F' episome is maintained by growth on M9/+Thi minimal medium. Single colonies can be picked and grown up in LB broth with no practical loss of the F' episome, however repeated passage must be avoided. This strain was used to grow and amplify the fUSE2 expression library.

SOLR™

e14-(*mcrA*), Δ(*mcrCB-hsdSMR-mrr*)171, *sbcC*, *recB*, *recJ*, *umuC*::Tn*5*(kan^r), *uvrC*, *lac*, *gyrA*96, *relA*, *thi*-1, *endA*1, λ^R, F'[*proAB*, *lacI*^{q}ZΔM15] Su- (nonsuppressing)

SOLR cells are provided with the SurfZAP vector kit. When SOLR host cells containing the amplified phagemid library are infected with M13 helper phage, the phagemid is replicated as single stranded DNA and is packaged into M13 particles. SOLR cells are a supO strain and so allow the replication of phagemid DNA, but not the expression of the helper phage proteins. Therefore, the helper phage genome is not replicated but is lost from the SOLR cells as the library is amplified. SOLR cells are cultured on LB agar containing 50µg/mL kanamycin.

BL21(DE3)

F-, *ompT*, *hsdS*$_B$ (r_B-m_B-), *gal, dcm,* (DE3)

In pET vectors, target genes are cloned under the control of strong bacteriophage T7 transcription and translation signals. Expression is induced by providing a source of T7 RNA polymerase in the host cell. Constructs are transferred into an expression host containing a chromosomal copy of the T7 RNA polymerase gene under *lacUV5* control, expression is induced by the addition of IPTG. BL21(DE3) carries a chromosomal copy of T7 RNA polymerase gene under the control of the *lacUV5* promoter. This makes it suitable for the production of protein from genes cloned in pET vectors.

MC1061

*araD*139, Δ(*ara*, *leu*)7697, Δ(*lac*)χ_{74}, *galU*, *galK*, *hsdR*2, *str*A, *mcrA*, *mrcB*1

This is a *rec*A+ strain that shows high electroporation efficiency and was used to produce the fUSE2 expression library.

Ligation

Ligation of restriction fragments was achieved using a Stratagene ligation kit according to the manufacturer's recommendations. Wherever possible forced directional cloning or dephosphorylated vectors were used. For blunt-end ligation dephosphorylated vectors were used and ten-fold more T4 DNA ligase was used in the reaction.

Restriction digestion

All restriction enzymes were used in accordance with the manufacturer's recommendations. Generally, material was digested with an excess of around 10-fold (i.e. 5U of enzyme per 1μg of DNA for 2 hours at the recommended temperature).

Phenol/chloroform purification

An equal volume of phenol:chloroform:isoamyl alcohol (Sigma, 25:24:1) was added to the nucleic acid sample in an eppendorf tube. The contents were mixed vigorously until an emulsion formed. The tube was centrifuged in a MSE microfuge at 13000rpm for 2 mins to separate the organic and aqueous phases. The top aqueous phase was transferred to a fresh tube. An equal volume of water was added to the organic phase and mixed vigorously. The phases were partitioned by centrifugation as described above. The top aqueous phase was removed and added to the aqueous phase from the first extraction. The phenol/chloroform extraction was repeated until no protein was visible at the solvent interface. An equal volume of chloroform was added to the nucleic acid sample and mixed vigorously until an emulsion formed, then centrifuged in a MSE microfuge at 13000rpm for 2 mins to separate the organic and aqueous phases. The top aqueous phase was transferred to a fresh tube and the lower organic phase and interface discarded. The DNA was recovered by precipitation with ethanol.

Ethanol precipitation

A 1/10 volume of 3M sodium acetate, pH5.3 was added to the nucleic acid and mixed. Exactly two volumes of ice-cold ethanol was added and mixed. The tube was stored on ice for two hours to allow the DNA to precipitate. The DNA was pelleted by centrifuging at 13000rpm in a MSE microfuge in a cold room. The supernatant was carefully aspirated and the tube half filled with 70% ethanol. The tube was spun at 13000rpm for 2 mins and the 70% ethanol aspirated. The tube was store open on the bench at room temperature until all fluid had evaporated. The DNA pellet was dissolved in an appropriate quantity of purified water.

Preparation of competent cells

A single *E.coli* colony was inoculated into 50mL of LB agar and agitated at 37°C until an absorbance of 0.5 at 600nm was reached. The culture was cooled on ice for 10 mins and spun at 2000g for 10 mins. The cell pellet was drained thoroughly then resuspended in a volume of RF1 that was 1/3 of the original culture volume. The cells were incubated for 15 mins on ice then pelleted as described above. The cell pellet was resuspended in a volume of RF2 that was 1/12.5 of the original culture volume. The cells were incubated on ice for 15 mins, then aliquoted in 100µL amounts in microfuge tubes and snap frozen. Competent cells were stored ready to use at -80°C for up to 4 weeks.

RF1	**Amount/litre**	**Final concentration**
RbCl	12g	100mM
$MnCl_2.4H_2O$	9.9g	50mM
1M Potassium acetate, pH7.5	30mL	30mM
$CaCl_2.2H2O$	1.5g	10mM
Glycerol	150g	15% (w/v)

Adjust to pH5.8 with 0.2M acetic acid and 0.2µm sterile filter.

RF2	**Amount/litre**	**Final concentration**
0.5M MOPS (pH6.8)	20mL	10mM
RbCl	1.2g	10mM
$CaCl_2.2H_2O$	11g	75mM
Glycerol	150g	15% (w/v)

Adjust to pH6.8 with NaOH and 0.2µm sterile filter.

Transformation

Cells were transformed with a maximum of 0.5ng of ligation reaction per µL of competent cells. The cells were thawed on ice and the ligation reaction added to the tube. The competent cell/ligation mixture was incubated on ice for 30 mins, then heat shocked at 42°C for 2 mins before returning to the ice for a further 5 mins. 500µL of warm LB broth was added and the culture incubated for 1 hour

at 37°C. The cells were plated on to LB agar plates containing the appropriate antibiotic for the vector being transformed using a sterile glass spreader. The plates were incubated overnight at 37°C in a warm air oven.

Agarose gel electrophoresis

Horizontal agarose gel electrophoresis was used to analyse and purify DNA fragments. The agarose concentration used depended on the size of fragments to be resolved. Concentrations of 0.7 and 2.5% were used. Agarose was dissolved in x1 TBE or TAE buffer by heating in a microwave, allowed to cool before ethidium bromide was added to 0.5µg/mL. The agarose was set in the electrophoresis tank tray and submerged in x1 TAE or TBE as appropriate. DNA was diluted in x6 Loading buffer, added to the well in the agarose gel in a horizontal submarine electrophoresis apparatus (Bio-Rad). The gel was run at no more than 5V/cm until the tracking dye reached the end of the gel. The DNA was visualised under UV illumination.

x6 Loading buffer	**Amount/Litre**
Bromophenol blue	2.5g
Glycerol	300mL

Dissolved in purified water and made up to 1 L.

x10 TBE buffer	**Amount/Litre**
Tris base	54g
Boric acid	27.5g
0.5M EDTA, pH8.0	20mL

Dissolved in purified water and made up to 1 L.

x50 TAE buffer	**Amount/Litre**
Tris base	242g
Glacial acetic acid	57.1mL
0.5M EDTA, pH8.0	100mL

Dissolved in purified water and made up to 1 L.

Purification of DNA

Gel purification of restricted DNA was used as a method to obtain fragments of interest for subsequent cloning. DNA was separated as described above using a 0.7% TAE low melting point gel containing no ethidium bromide. The gel was stained in a 0.5µg/mL ethidium bromide solution for 30 mins, then destained for 30 mins in water before visualising the DNA under UV illumination. The band of interest was excised from the gel with a scalpel blade and placed in a pre-weighed microfuge tube. The DNA was purified using a Qiagen DNA Gel Extraction Kit according to the manufacturer's instructions followed by ethanol precipitation. The DNA was redissolved in a suitable volume of purified water.

Polymerase chain reaction

The polymerase chain reaction (PCR) was used to amplify DNA sequences used in the generation of various plasmid constructs detailed later.

The primers were designed specifically to meet the needs of each amplification. Their melting temperatures were calculated using the formula; Tm(°C)=64.9+0.41(%GC)-600/N

The PCR was setup in 0.5mL eppendorf tubes as follows:

Template DNA	~ 0.1pmol
Forward primer (10µmol/L)	5µL
Reverse primer (10µmol/L)	5µL
x10 PCR buffer (Gibco BRL)	5µL
50 mmol/L $MgCl_2$	1.5µL
10 mmol/L dNTPs	1µL
Taq polymerase (Gibco BRL)	0.5µL

The volume was made up to 50µL with purified water and 50µL of mineral oil was placed on the top to prevent evaporation during the thermal cycling. The reactions were placed in a PTC-100 thermal cycler (MJ Research) and denatured for 5 minutes at 93°C, followed by 30 cycles of 60 secs at 93°C, 60 secs at Tm-5°C and 90 secs at 72°C.

The thermal cycle was modified for primers designed with a non-complementary 5' end. A lower annealing temperature corresponding to the Tm of the complementary region of the primers was used for 5 cycles, followed by 30 cycles at an annealing temperature suitable for the whole primer.

Purification of M13 phage particles

10mL of culture was spun at 6000g for 10 mins and the phage containing supernatant transferred to a fresh tube. 200µL of 30%PEG/2.5M NaCl was added for every 1mL of supernatant, mixed well by inversion and incubated at room temperature for 15 mins. The precipitate was pelleted by centrifuging at 10,000g for 10 mins and the supernatant aspirated and discarded. The pellet was resuspended in 1mL of TE buffer, transferred to a microfuge tube and spun at 10,000 rpm in a MSE microfuge for 10 mins to pellet the cell debris. The supernatant was transferred to a fresh microfuge tube and the PEG precipitation repeated. The final phage pellet was resuspended in 100µL of TE buffer and stored at 4°C.

Phage titration

Suitable plating bacteria were prepared by the method described in Sambrook *et al.* (1989). The phage suspension was 10-fold serially diluted in TE buffer. 1µL-10µL of diluted phage was added to 100µL of plating bacteria and incubated at room temperature for 5 mins to allow the phage to attach to the cells. The cells were then spread on to LB agar plates containing a suitable antibiotic complementary to the phage resistance marker and incubated overnight at 37°C.

Lambda phage was titrated by plaque counting. The phage suspension was 10-fold serially diluted in SM buffer. 1µL-10µL of diluted phage was added to 100µL of plating bacteria and incubated at 37°C for 15 mins to allow the phage to attach to the cells. 3mL of melted NZY top agar was added to each sample and the agar immediately poured on to warm NZY plates. The top agar was allowed to set. The plates were inverted and incubated for 6-8 hours at 37°C for plaques to become visible.

The titre (cfu/mL or pfu/mL) was determined by counting the number of colonies or plaques formed and using the formula;

Titre(cfu/mL or pfu/mL) = (number of colonies x dilution factor x 1000) / phage volume plated (µL)

SDS-PAGE

SDS-polyacrylamide electrophoresis was used to separate protein mixtures according to relative molecular weight to assess them qualitatively and semi-quantitatively. Samples were electrophoresed using a Mini-protean II apparatus (Bio-Rad) according to manufacturer's instructions, which are based on the method of Laemmli (1970). Briefly, samples were diluted in sample buffer and 5µL loaded into each lane of a 12% gel and electrophoresed at 150v until the tracker dye reached the bottom of the gel. The gel was removed from the glass plates and stained with Coomassie R-250 stain for 30mins, then destained with repeated changes of destain solution until the background was clear. They were then dried onto blotting paper using a vacuum gel drier set at 80°C for 2 hours.

Sample buffer	**Amount**
Purified water	4.0mL
0.5M Tris-HCl, pH6.8	1.0mL
Glycerol	0.8mL
10% (w/v) SDS	1.6mL
2-βmercaptoethanol	0.4mL
0.05% (w/v) Bromophenol blue	0.2mL

Samples were diluted at least 1:4 with sample buffer and heat at 95°C for 4 mins

Running gel (12%)	Amount
Purified water	3.35mL
1.5M Tris-HCl, pH8.8	2.5mL
10% (w/v) SDS	100µL
37.5:1 Acrylamide/Bis (30% stock)	4.0mL
10% (w/v) Ammonium persulphate	50µL
TEMED	5µL

Stacking gel (4%)	Amount
Purified water	6.1mL
0.5M Tris-HCl, pH6.8	2.5mL
10% (w/v) SDS	100µL
37.5:1 Acrylamide/Bis (30% stock)	1.6mL
10% (w/v) Ammonium persulphate	50µL
TEMED	10µL

Electrode buffer (x5), pH8.3	Amount/Litre
Tris base	15g
Glycine	43.2g
SDS	3g

60mL x5 stock was diluted with 240mL of purified water to make sufficient buffer for one electrophoresis run.

Stain	Amount/Litre
Coomassie Brilliant Blue R250	2.5g
Methanol	450mL
Water	450mL
Glacial acetic acid	100mL

Destain	Amount/Litre
Methanol	200mL
Water	725mL
Glacial acetic acid	75mL

Western blotting

Samples from SDS-polyacrylamide gels were transferred onto nitrocellulose using a Trans-Blot apparatus (Bio-Rad) according to the manufacturer's instructions. Briefly, gels were laid onto wetted Whatman 3M filter paper and a sheet of Hybond-C nitrocellulose (Amersham) applied to the upper surface, followed by another sheet of 3M paper. This was sandwiched between two porous pads and submerged in transfer buffer in the Trans-Blot tank. The proteins were transferred overnight at 0.1mA.

Transfer buffer (pH8.3)	Amount/litre	Final concentration
Glycine	2.9g	39mM
Tris base	5.8g	48mM
SDS	0.37g	0.037% (w/v)
Methanol	200mL	20% (v/v)

The nitrocellulose was remove from the gel and blocked by incubating in PBS containing 0.05% Tween 20 and 1% (w/v) dried milk powder for 1 hour at room temperature. The specific primary antibody was diluted in the same solution and the nitrocellulose incubated for 1 hour with gentle agitation. The primary antibody was aspirated and the nitrocellulose washed four times with PBS containing 0.05% Tween 20 by sequential buffer changes. The second antibody, a species specific anti-Ig antibody labeled with horseradish peroxidase, was diluted 1:1000 in PBS containing 0.05% Tween 20 and 1% (w/v) dried milk powder and incubated with the nitrocellulose for 1 hour with gentle agitation. The nitrocellulose was washed four times as previously described. The blot was developed using the substrate 4-chloro-1-napthol in the form of the HRP Substrate Kit (Bio-Rad). It was made to working strength as described in the manufacturer's instructions, added to the nitrocellulose membrane and allowed to develop to the required colour intensity. The reaction was stopped by decanting the substrate and washing the membrane several times with purified water. The resulting banding patterns were analysed using GelCompar™ (Applied Maths, Kortrijk) electrophoresis analysis software where appropriate.

Serum panels

A panel of disease stage characterised serum specimens, consisting of 18 patients classified with early primary syphilis, 36 patients with late primary or secondary syphilis, 18 patients with latent syphilis, 20 patients with successfully treated infections, and 12 patients confirmed as biological false positives was obtained from Dr. S. I. Egglestone (Bristol Public Health Laboratory, UK). These specimens were classified by the supplier using a combination of clinical presentation and the results of VDRL, TPHA, and FTA-ABS results, which were supplied with the specimens.

A panel of 7 Lyme disease positive sera was obtained with testing data from a commercial supplier, Boston Biomedical (Boston, MA).

A panel of serum specimens consisting of 25 patients with well established infections or reinfections, characterised by high antibody titres and 76 non-selected serum specimens positive for syphilis from a genito-urinary clinic were obtained from Dr. D. J. Merry (Institute of Medical and Veterinary Science, Adelaide, South Australia). These specimens were supplied with limited clinical data and serological testing results, and so were tested using the Captia® Syphilis-G test according to the manufacturer's instructions.

Serum specimens from healthy blood donors and antenatal patients, with no history of syphilis, were obtained from North London Blood Transfusion Centre. Although some testing data was provided, the specimens were tested using the Captia® Syphilis-G test according to the manufacturer's instructions.

Antisera

Rabbit antisera specific for *T.pallidum* was supplied from archived sources by Dr. J. A. Carney (Centocor UK Ltd) and Prof. C. W. Penn (University of Birmingham). Before use it was tested by western blotting against antigen derived from whole *T.pallidum* organisms to confirm the activity had not decayed on storage, and the claimed specificity was correct.

Cloning and expression of the major lipoproteins

pET10

This construct was provided by Ms. A. Ivic and Prof. C. W. Penn, University of Birmingham. It consists of the whole *tpn*44.5 gene inserted between the *NdeI/NotI* sites in the multiple cloning site of the pET21b vector.

pET17

This construct was provided by Ms. A. Ivic and Prof. C. W. Penn, University of Birmingham. It consists of the whole *tpn*17 gene inserted between the *NdeI/NotI* sites in the multiple cloning site of the pET21b vector.

pET50

This construct was provided by Ms. A. Ivic and Prof. C. W. Penn, University of Birmingham. It consists of the whole *tpn*24-28 gene inserted between the *NdeI/NotI* sites in the multiple cloning site of the pET21b vector.

pET50REP

This construct was provided by Ms. A. Ivic and Prof. C. W. Penn, University of Birmingham. It consists of the *tpn*24-28 gene, excluding the upstream acetylation sequence, inserted between the NdeI/NotI sites in the multiple cloning site of the pET21b vector.

pET60

This construct was provided by Ms. A. Ivic and Prof. C. W. Penn, University of Birmingham. It consists of the whole *tpn*15 gene inserted between the *Nde*I/*Not*I sites in the multiple cloning site of the pET21b vector.

pET60REP

This construct was made by PCR amplification of *tpn*15, excluding the upstream acetylation sequence, using the primers 5' GTTGCTTGGGCATATGTCATTTAGTTC 3' and 5' GCGCGGCCGCCCTGCTAATAATGGCTTCC 3'. The PCR product was NdeI/NotI digested, gel purified and the DNA recovered using Qiagen Gel Extraction kit followed by ethanol precipitation. The DNA was ligated with suitably digested pET21b vector DNA (Novagen), transformed into competent XL-1Blue *E.coli* cells and plated on to LB agar containing 100µg/mL ampicillin. Transformants were screened for the correct insert by restriction digestion and agarose electrophoresis. The vector/insert junctions were checked by DNA sequence analysis.

LIC47

This construct was made by PCR amplification of *tpn*47, excluding the upstream acetylation sequence, using the primers 5' GACGACGACAAGATGGGCTCGTCTCATCATGAG 3' and 5' GAGGAGAAGCCCGGTCACTGGGCCACTACC 3' which also modify the ends of the amplified fragment to incorporate the LIC cloning sequences. The PCR product was purified using Qiagen PCR purification kit and ethanol precipitated. The DNA was treated with T4 DNA polymerase as described in the manufacturer's instructions and annealed with pre-treated pET32LIC vector DNA (Novagen). The sample was transformed into competent XL-1Blue *E.coli* cells and plated on to LB agar containing 100µg/mL ampicillin. Transformants were screened for the correct insert by restriction digestion and agarose electrophoresis. The vector/insert junctions were checked by DNA sequence analysis.

LIC15

This construct was made by PCR amplification of *tpn*15, excluding the upstream acetylation sequence, using the primers 5 GACGACGACAAGATGTGTTCATTTAGTTCT 3' and 5' GAGGAGAAGCCCGGTTTCTACCTGCTAATA 3'. These primers modify the ends of the amplified fragment to incorporate the LIC cloning sequences and introduce an amber stop codon. The PCR

product was purified using Qiagen PCR purification kit and ethanol precipitated. The DNA was treated with T4 DNA polymerase as described in the manufacturer's instructions and annealed with pre-treated pET32LIC vector DNA (Novagen). The sample was transformed into competent XL-1Blue *E.coli* cells and plated on to LB agar containing 100µg/mL ampicillin. Transformants were screened for the correct insert by restriction digestion and agarose electrophoresis. The vector/insert junctions were checked by DNA sequence analysis.

Protein expression

The recombinant plasmid was transformed into BL21(DE3) *E.coli* cells and plated for single colonies on LB agar containing 100µg/mL ampicillin. In a 250 mL conical flask, 50mL of LB broth containing 100µg/mL ampicillin was inoculated with a single colony and grown at 37°C in a shaker incubator until the A_{600} of the culture reached 0.6. Samples were taken as uninduced controls for examination by SDS-PAGE. IPTG was added to a final concentration of 1mM and the culture grown for a further 2 hours. The flask was cooled on ice for 5 mins and then the cells were harvested by centrifugation at 6000g for 10 mins. The supernatant was discarded and the pellet resuspended in ice cold 50mM Tris, pH8.0 buffer containing 1mM PMSF.

Protein purification

The cell pellet was kept on ice throughout the procedure. It was sonicated at power level 4 for 10 cycles of 30 seconds on followed by 30 seconds rest using an Ultrasonix sonicator fitted with a microtip probe. The disrupted pellet was centrifuged at 10,000g for 10 mins, the supernatant discarded and the pellet resuspended in 10mM Tris, pH8.0 containing 6M guanidine.HCl. It was then respun at 10,000g for 10 mins and the supernatant transferred to a clean tube. 5mL of equilibrated Talon metal affinity resin (Clontech) was added and mixed on a roller mixer for 20 mins. The resin was spun at 700g for 5 mins and the supernatant aspirated. 50mL of 10mM Tris, pH8.0 containing 8M urea was added and the tube mixed on a roller mixer for 10 mins. The tube was centrifuged at 700g for 5 mins and the supernatant aspirated. This wash procedure was repeated three more times, then 5mL of 10mM Tris, pH8.0 containing 8M urea and 100mM imidazole was added and mixed on a roller mixer for 10 mins. The tube was centrifuged at 700g for 10 mins and the supernatant, containing eluted recombinant protein, aspirated and transferred to a

fresh tube. This elution procedure was repeated three more times. The pre and post induction samples, and the four elution samples were analysed by SDS-PAGE.

Preparation of affinity purified antisera

Affinity column preparation

Purified recombinant TpN47, TpN44.5, TpN17 and TpN15 and a lysate from *E.coli* BL21(DE3) cells were dialysed into 0.2M $NaHCO_3$, 0.5M NaCl, pH8.3 and the protein concentration estimated using a Bradford dye binding assay (Bio-Rad) following manufacturer's recommendations. 10mg of each of the proteins was coupled to separate 1 mL NHS-HiTrap columns (Pharmacia) following the manufacturer's recommendations using FPLC®. The efficiency of coupling was monitored by measuring the A_{280} of the protein solution before and after coupling as recommended by the manufacturer's. The coupling efficiencies ranged between 78% and 92%.

Affinity purification of antisera

The five affinity columns were coupled together in series with the *E.coli* lysate column first and connected to an FPLC® system. A 500mL pool of syphilis positive human sera was diluted with an equal volume of PBS, 0.2µm filtered and applied to the columns at 1mL/min. The flow from the column was collected for reuse. The columns were washed with 25-30mL of PBS or until the A_{280} trace returned to baseline. The columns were separated and eluted individually with 5mL of 0.2M glycine/HCl, pH2.2 which was collected in 0.5mL fractions. All protein containing fractions were pooled and 150µL of 1M Tris/HCl, pH9.1 was added per mL of protein containing solution.

A second purification of the serum pool was conducted as described above and the eluted protein pooled with that obtained from the first purification run.

Enzyme immunoassay (EIA)

Plate coating

The coating concentration was optimised for each material to be immobilised. Simple coatings of single proteins were optimised by coating curve analysis. A range of concentrations of the material were coated under various conditions, and tested for functional activity using the chosen EIA format. The coating concentration was selected on the basis of the best compromise of a series of

criteria; concentration, total activity, background signal, and dose response. Coatings of protein mixtures, where interactions between components complicate the picture, were optimised using statistical experimental design software (ECHIP version 6.04) to formulate and perform response surface analysis of a multifactorial mixture design. The optimal coating was selected using similar criteria to those applied to simple single protein coatings. The material to be immobilised was diluted to the optimal coating concentration, usually between 1 and 10µg/mL, in 0.01M carbonate buffer (pH9.6). It was added at 100µL per well to Maxisorp 96-well microplates (Nunc), covered to prevent evaporation and incubated for 18 hours at room temperature. The wells were washed three times with PBS containing 0.05% Tween 20, then filled with a 5% (w/v) lactose containing 1% (w/v) dried milk powder and allowed to stand for 5 minutes. The lactose/dried milk powder solution was aspirated and any residual solution removed by inversion onto adsorbent paper towels. The plates were dried for 2 hours in a 37°C warm air oven, sealed in a foil pouch with a desiccant sachet and stored at 4°C until required.

Assay procedure

Samples to be tested were diluted 1:21 in PBS, pH7.4 containing 0.05% Tween 20 and 1% dried milk powder. 100µL of diluted sample was added to each well, the plate was sealed to prevent evaporation and incubated at 37°C for 1 hour. The plate was washed five times with PBS containing 0.05% Tween 20 and blotted dry on to adsorbent paper towels. Monoclonal anti-human IgG/horseradish peroxidase conjugate (Sigma I-2067) was diluted 1:15000 in PBS, pH7.4 containing 0.05% Tween 20. 100µL of diluted conjugate was added to each well, the plate was sealed to prevent evaporation and incubated at 37°C for 1 hour. The plate was washed five times with PBS containing 0.05% Tween 20 and blotted dry on to adsorbent paper towels. Working strength substrate was prepared by adding 25µL of TMB chromogen to each 1mL of substrate solution. 100µL of working strength substrate solution was added to each well and incubated at room temperature for 30 mins. The enzyme reaction was stopped by adding 100µL of 0.25M H_2SO_4 to each well, mixed and the adsorbance read at 450 nm using a Multiskan plate reader (Labsystems).

Substrate solution	Amount
Glacial acetic acid	2.2g
Sodium acetate.$3H_2O$	1.8g
Disodium EDTA.$2H_2O$	1.5g
Sodium perborate.$3H_2O$	0.74g
Purified water	1000g
Target pH 4.47	

TMB chromogen	Amount
Dimethylsulphoxide (sg ~1.1)	110g
3,3'5,5'-tetramethylbenzidine	1g
Eosin B (spirit soluble)	0.02g

Synthetic peptide epitope mapping

Peptide synthesis

The amino acid sequences of TpN47, TpN44.5, TpN17 and TpN15 were determined from the DNA sequences held on the Genebank database and used to design sets of overlapping peptides to cover each of the sequences. TpN47 was mapped using 101 14-mer peptides offset by 4 aa with an overlap of 8 aa covering the mature protein sequence from residue 21 through to residue 434 at the C-terminus. TpN44.5 was mapped using 79 14-mer peptides offset by 4 aa with an overlap of 8 aa, except the last peptide which was offset by only 1 aa. The peptides covered the sequence from residue 23 to final residue, 345. TpN17 was mapped using 42 12-mer peptides offset by 3 aa with an overlap of 9 aa covering the sequence from residue 22 through to residue 176. TpN15 was mapped using 58 10-mer peptides offset by 2 aa with an overlap of 8 aa covering the sequence from residue 18 to the end at residue 141. These peptides along with control peptides, PLAQ (positive) and GLAQ (negative), were synthesized using commercially available epitope mapping system (Chiron Mimotopes Pty) according to the manufacturer's instructions. The completion of each successive amino acid coupling was confirmed by the presence of bromophenol blue in the reaction.

Testing

200µL/well of PBS containing 0.1% Tween20 and 2% (w/v) BSA was dispensed into a microplate. The pins were inserted into the plate and incubated for 1 hour at room temperature on a rotating platform (100rpm). The pins were removed and washed once in a bath of PBS for 10mins on a rotating platform (100rpm). The test antibody was diluted 1:1000 in PBS and 200µL/well dispensed into the wells of a microplate. The pins were inserted into the wells, sealed in a box to prevent evaporation and incubated overnight at room temperature on a rotating platform (100rpm). The pins

were washed four times in a bath of PBS for 10 mins per wash using fresh PBS each time. Monoclonal anti-human IgG/horseradish peroxidase conjugate (Sigma I-2067) was diluted 1:5000 in PBS, pH7.4 containing 0.05% Tween 20 and 200µL/well dispensed into a microplate. The pins were inserted and incubated for 1 hour at room temperature on a rotating platform (100rpm), then washed four times as previously described. 150µL/well of working strength substrate was dispensed into the wells of a microplate and the pins inserted. The colour development reaction was allowed to proceed for 30 mins before being stopped by the addition of 150µL of 0.25M H_2SO_4. The well contents were mixed and their absorbances read at 450nm using a Multiskan plate reader (Labsystems).

Removal of antibody

The blocks of pins were immersed in PBS containing 1% (w/v) SDS and 0.1% (v/v) 2-βmercaptoethanol in a sonicator bath, preheated to 60°C, and sonicated for 10 mins. The pins were rinsed twice in purified water, preheated to 60°C, for 30 seconds. Then they were washed for 30 minutes in a bath of purified water at an initial temperature of 60°C on a rotating platform. The pins were shaken dry and immersed in a bath of methanol, preheated to 60°C, for 15 seconds. The pins were air dried for at least 15 mins before being ready for another test. Alternatively, the pins were sealed in a foil bag with desiccant at 4°C until required.

PCR synthesized random gene fragment library

Overview

The method below describes the construction of four separate random gene fragment libraries, one for each of the four major lipoproteins of *T.pallidum,* in the SurfZAP vector system (Stratagene). The SurfZAP vector encodes the N-terminal portion of the pelB leader sequence, upstream of the NotI restriction site. Therefore, the 5' end of the cloned inserts must include the remainder of the pelB DNA sequence located between the NotI site and the N-terminus of the fusion protein. The 3' end of the DNA inserts were designed to be in-frame with the spacer-gene III sequence and included the *Spe*I restriction site. Suitable inserts were prepared by a nested PCR method. DNA sequences corresponding to the *tpn*47, *tpn*44.5, *tpn*17 and *tpn*15 genes were obtained by PCR amplification. This DNA was used as the template for an amplification reaction using primers with 10 random bases at the 3' end, which at a low annealing temperature allows for random priming.

The DNA from this reaction was PCR amplified using primers complementary to just the 5' end of the original primers. The amplified DNA was ligated into the SurfZAP vector and converted into phage particles using helper phage. The recombinant phage particles display random polypeptide sequences as fusion proteins at the N-terminus of the cpIII protein. This gene-targeted random epitope library was screened by affinity selection for recombinant phage which were specifically bound by the antibody of interest.

Library construction

Genomic DNA was isolated from *T.pallidum* subsp. *pallidum* (Nichols strain) using IsoQuick DNA extraction kit according to the manufacturer's instructions. The genomic DNA was used as the template in PCRs to obtain separate samples of *tpn*47, *tpn*44.5, *tpn*17 and *tpn*15 DNA. The primers used are shown below.

Primer	Designation	Sequence	Tm
*tpn*47 forward	47S	5' CGGAGGTGTACATATGAAAGTGAAATACGC 3'	62°C
*tpn*47 reverse	47T	5' ACACCCCTCTCGAGCTGGGCCACTA 3'	67°C
*tpn*44.5 forward	TmpA1	5' GCGCATATGAATGCTCATACGCTTGTGTAC 3'	64°C
*tpn*44.5 reverse	TmpA2	5' GCGCGGCCGCTCGAGAGGCTCCTTCTTCGT 3'	74°C
*tpn*17 forward	SP8PROM	5' GCGCATATGAAAGGATCTGTCCGCGCG 3'	66°C
*tpn*17 reverse	SP8TERM	5' GGGGCGGCCGCTTTCTTTGTTTTTTTGAGC 3'	67°C
*tpn*15 forward	60P2	5' GCGGCACTCGGAGGTCATATGGTG 3'	65°C
*tpn*15 reverse	60T	5' GCGCGGCCGCCCTGCTAATAATGGCTTCC 3'	71°C

The reaction was run for 30 cycles of 93°C for 60 secs, 60°C for 60 secs and 72°C for 90 secs. The PCR products were purified using Qiagen PCR purification kit according to the manufacturer's instructions.

Generation of inserts

Each of the DNAs were used in separate PCRs to generate separate sets of random gene fragments. Sixteen PCRs were set up using the primers 5' CTCGCTCGCCCATATGCGGCCGCAGGTCTCCTCCTCTTAGCAGCACAACCAGCAATGGCCXX XXXXXXXX 3' and 5' GCCGGCCCGCCCAGCATCACTAGTXXXXXXXX 3' where X represents a random base. The reactions were setup to the standard composition, except much more template was used in each reaction compared to a normal PCR. Template to primer ratios of 1:1, 1:2, 1:5 and 1:10 were used. The reactions were run for five cycles of 93° for 60 secs, 20°C for 60 secs and 72°C for 90 secs. The four reactions from each gene were pooled and purified using Qiagen PCR purification kit to remove unused primers and primer dimers. The purified DNA was used as the

template for a second PCR using primers 5' CTCGCTCGCCCATATGCGGCCGCA 3' and 5' GCCGGCCCGCCCAGCATCACTAGT 3'. The reaction was cycled 93°C for 60 secs, 60°C for 60 secs and 72°C for 90 secs for 35 cycles. The reaction was purified using Qiagen PCR purification kit.

Restriction digestion, ligation and packaging

The purified DNA was digested with an excess of *Not*I and *Spe*I restriction enzymes and ligated with pretreated SurfZAP lambda arms (Stratagene) with positive and negative controls. The DNA from the ligation reaction was packaged directly using Gigapack II Plus packaging extract (Stratagene) and plaque titrated on NZY plates using XL1-Blue *E.coli* cells.

Characterisation of library

10 discrete plaques were randomly cored from a titration plate and eluted into 1mL of SM buffer. The suspension was spun at 10,000g for 10 mins in a microfuge to pellet any debris and resuspended in SM buffer. The phage stock was used as the template in a PCR designed to amplify across the cloning site of the vector. The upstream primer was (T3 promoter) 5' ATTAACCCTCACTAAAGGGAA 3' and the downstream primer (T7 promoter) 5' CCCTATAGTGAGTCGTATTA 3'. The components of the reaction were as previously described and the thermal cycle was 25 cycles of 93°C for 60 secs, 50°C for 60 secs and 72°C for 90 secs. A 5µL aliquot of each reaction was electrophoresed through a 2.5% agarose gel containing 0.5µg/mL ethidium bromide and visualised under UV illumuination. A modification of this method was also used to determine the size of the inserts in a selection of colonies isolated after biopan enrichment of the library. The modification being that the template used was pSurfscript SK- plasmid DNA isolated by a miniprep method rather than lambda phage particles.

Amplification

The phage (10^5 pfu) were mixed with 100µL of plating bacteria and incubated at 37°C for 20 mins. 3mL of molten (50°C) NZY top agar was added and mixed. The agar was poured onto a warmed NZY agar plate and swirled to spread. It was allowed to cool before being inverted and incubated at 37°C for 8 hours. 5mL of SM buffer was added to each plate and stored at 4°C for 2 hours with gentle agitation. The liquid was harvested and a further 1mL of SM buffer added and agitated for 15 mins. The plates were inclined to allow the fluid to drain and as much SM buffer as possible to be

harvested. The SM buffer was spun at 4000g for 10 mins and the supernatant aspirated into a sterile tube. DMSO was added to 7% (v/v) and stored at -80°C. The number of phage was estimated by plaque titration on NZY plates using XL1-Blue *E.coli* cells. This represents the amplified lambda libraries.

Mass excision and amplification

In a 50mL conical tube, SurfZAP bacteriophage and XL1-Blue were combined at a mutiplicity of infection of 1:10 lambda phage to cell ratio. ExAssist helper phage was added at a 1:1 helper phage to cell ratio. The mixture was incubated for 15 mins at 37°C, then 20mL of LB broth was added and the tube incubated with gentle agitation for 3 hours at 37°C. The culture was placed in a water bath heated to 70°C for 20 mins to lyse the lambda phage particles and the XL1-Blue cells. The tube was centrifuged at 2500g for 10 mins to pellet the debris and the supernatant transferred to a fresh tube. The excised phagemid particles were stored at 4°C. The number of particles (cfu) excised was determined by titration on LB agar plates containing 100µg/mL ampicillin. The excised phagemids were amplified in SOLR *E.coli* cells to eliminate the ExAssist helper phage genome from the library. SOLR *E.coli* cells were mixed with the excised supernatant so that there was at least one cell per phagemid. The cells were incubated with the phagemids at 37°C for 15 mins. 100mL of LB broth containing 100µg/mL carbenicillin, 50µg/mL kanamycin and 1% (w/v) glucose was added and the culture incubated with shaking at 37°C until an A_{600} of 1.0 was reached. The culture was centrifuged at 1000g for 10 mins to pellet the cells. The cells were resuspended in 10mM $MgSO_4$ and stored overnight at 4°C.

Conversion to phage particles

The SOLR *E.coli* cells were grown to an A_{600} of 0.2, centrifuged and 10^9 cells resuspended in 1mL of LB broth. 10^{10} VCSM13 helper phage were added and incubated at 37°C for 15 mins. 10mL of LB broth containing 100µg/mL carbenicillin and 50 µg/mL kanamycin was added and the culture grown at 37°C with gentle agitation until an A_{600} of 1.0 was reached. The culture was centrifuged at 2500g for 10 mins and the supernatant transferred to a fresh tube. The phagemid particles were purified by two PEG precipitations and finally resuspended in 1mL of TE buffer. The titre was determined by titration on to LB agar plates containing ampicillin. This represents a library of

phagemid particles expressing the cpIII fusion protein on the surface and containing the recombinant cpIII fusion protein DNA sequence.

Affinity selection of phage displayed peptides

Purified IgG (various) was diluted to 10µg/mL in 10mM sodium carbonate buffer, pH9.6 and 1mL dispensed in to a Maxisorp Immunotube (Nunc). The tube was capped and incubated at room temperature for 18 hours. It was washed three times with PBS containing 0.05% Tween 20 using a wash bottle. Excess wash fluid was removed using a disposable pipette. The tube was blocked by adding 1mL of PBS containing 2% dried milk powder and incubated at room temperature for 1 hour. Approximately 10^{10} library phage were added to a microfuge tube containing 1mL of PBS supplemented with 2% dried milk powder, 0.1%(v/v) E.coli/phage lysate (Stratagene) and 1µg/mL purified human IgG. The phage was incubated at 37°C for 1 hour. The blocking solution was aspirated from the coated tube and replaced with the diluted phage solution, mixed and incubated at 37°C for 3 hours with agitation. The tube was filled with PBS containing 0.05% Tween 20 using a wash bottle and thoroughly aspirated using a disposable pipette. This wash procedure was repeated a total of ten times. The bound phage was eluted by adding 1mL of 0.2M glycine/HCl buffer, pH2.2 for a maximum of 10 mins. The eluted phage were aspirated into a sterile universal, neutralised by adding 150µL of 1M Tris, pH9.1 and 200µL of XL1-Blue plating bacteria was added. An aliquot of the eluted phage was retained for quantitation. The universal was agitated for 15 mins at 37°C to allow the phage to attach to the cells before 9mL of LB broth containing 100µg/mL carbenicillin was added. The culture was incubated for 1 hour at 37°C. 10^{10} VCSM13 helper phage were added before incubating for 1 hour at 37°C. Kanamycin was added to a final concentration of 50 µg/mL and the culture incubated overnight at 30°C. It was spun at 6000g for 10 mins and the phage purified from the supernatant by two PEG precipitations. The eluted phage and the purified phage were titrated with XL1-Blue cells on LB agar containing 100µg/mL ampicillin.

The biopanning procedure described above was repeated twice more, each time using the phage from the previous round as the starting material. In each successive round of biopanning the stringency of the wash protocol was increased. The second round was washed 15 times with PBS containing 0.1% Tween 20 and the third round was washed 20 times with PBS containing 0.5% Tween 20.

DNase1 digested random gene fragment library

Overview

DNA sequences corresponding to the *tpn*47, *tpn*44.5, *tpn*17 and *tpn*15 genes were obtained by PCR amplification. The pooled DNA was randomly digested with DNase1 to generate a population of DNA fragments of different sizes and sequences. After size fractionation, small DNA fragments (50bp - 350bp) were isolated and cloned into the phage expression vector fUSE2 to form an expression library displaying random polypeptide sequences as fusion proteins at the N-terminus of the phage gene III protein. The fUSE2 phage display expression vector (Parmley and Smith, 1988) was supplied by Dr. D. H. du Plessis, Onderstepoort Veterinary Institute, South Africa. This gene-targeted random epitope library was screened by affinity selection for recombinant phage which were specifically bound by the antibody of interest.

Library construction

Genomic DNA was isolated from *T.pallidum* subsp. *pallidum* (Nichols strain) using IsoQuick DNA extraction kit according to the manufacturer's instructions. The genomic DNA was used as the template in PCRs to obtain separate samples of *tpn*47, *tpn*44.5, *tpn*17 and *tpn*15 DNA. The primers used are shown below.

Primer	Designation	Sequence	Tm
*tpn*47 forward	47S	5' CGGAGGTGTACATATGAAAGTGAAATACGC 3'	62°C
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*tpn*44.5 forward	TmpA1	5' GCGCATATGAATGCTCATACGCTTGTGTAC 3'	64°C
*tpn*44.5 reverse	TmpA2	5' GCGCGGCCGCTCGAGAGGCTCCTTCTTCGT 3'	74°C
*tpn*17 forward	SP8PROM	5' GCGCATATGAAAGGATCTGTCCGCGCG 3'	66°C
*tpn*17 reverse	SP8TERM	5' GGGGCGGCCGCTTTCTTTGTTTTTTTGAGC 3'	67°C
*tpn*15 forward	60P2	5' GCGGCACTCGGAGGTCATATGGTG 3'	65°C
*tpn*15 reverse	60T	5' GCGCGGCCGCCCTGCTAATAATGGCTTCC 3'	71°C

The reaction was run for 30 cycles of 93°C for 60 secs, 60°C for 60 secs and 72°C for 90 secs. The PCR products were purified using Qiagen PCR purification kit according to the manufacturer's instructions.

A sample of the purified PCR products was electrophoresed through a 0.7% agarose gel containing 0.5µg/mL ethidium bromide, examined and quantitated under short wavelength UV illumination. The four DNA species were pooled in equimolar proportions. A trial DNase1 digestion was performed to determine to optimal conditions required to produce fragment of the desired size. DNase1 (Promega RNase-free, DNase 1000U/mL) was double diluted from 10U/mL to 0.3125U/mL in

DNase1 buffer (50mM Tris buffer, pH 7.6 containing 1mM $MnCl_2$). 1µg of DNA was combined with 1µL of x10 DNase1 buffer, 1µL of 1mg/mL acetylated BSA and made up to 10µL with purified water. All tubes were chilled on ice. 3.5µL of each diluted DNase1 was added to separate tubes, mixed and transferred a 15°C waterbath for exactly 10 mins. The digestion was stopped by adding 2µL of stop solution (70% glycerol, 75mM EDTA, 0.3% bromophenol blue) to each tube. The contents of each tube was electrophoresed through a 2.5% agarose gel containing 0.5µg/mL ethidium bromide and visualised under UV illumination.

DNase1 concentrations of 1.25U/mL and 2.5U/mL were selected to digest the bulk of the DNA. Reactions were set up each containing 30mg of pooled DNA dissolved in DNase1 buffer. 30µL x10 DNase1 buffer, 30µL 1mg/mL acetylated BSA and purified water to 300µL. All reactions were chilled on ice. 105µL of DNase1 diluted in DNase1 buffer was added and the reactions transferred to a 15°C waterbath for exactly 10 mins. The digestion was stopped by adding 30µL of 250mM EDTA. 1mg of DNA was removed from each reaction and electrophoresed through a 2.5% agarose gel containing 0.5µg/mL ethidium bromide and visualised under UV illumination to determine if the scale-up digestion had performed as predicted. The two digestions were pooled and the DNA purified by phenol/chloroform extraction and recovered by ethanol precipitation.

The DNA was dissolved in water and combined in an eppendorf tube with 250U of T4 DNA polymerase (Gibco BRL), dNTPs to a final concentration of 100µM each, DTT to a final concentration of 500µM and x5 T4 DNA polymerase buffer (Gibco BRL). The reaction was mixed and incubated in a 15°C waterbath for 15 mins. 10U of Klenow polymerase (large fragment) (NEB) was added and the reaction incubated for a further 15 mins at room temperature. The DNA was purified by phenol/chloroform extraction and recovered by ethanol precipitation. The DNA fragments were dissolved in water and combined into a ligation reaction with 200U of T4 DNA ligase (Stratagene), 40µg annealed 5' phosphorylated *Bgl*II linker (NEB), x10 T4 ligase buffer (Stratagene), rATP (Stratagene) to a final concentration of 500µM and made up to 1mL with water. The reaction was incubated overnight at 4°C. The DNA was phenol/chloroform extracted and recovered by ethanol precipitation. It was combined into a restriction digestion with 800U of *Bgl*II (NEB), x10 NEB-3 buffer, acetylated BSA to a final concentration of 100µg/mL, made up to 1mL with water and incubated at 37°C for 3 hours. A further 200U of enzyme was added and the

reaction incubated for another hour. The DNA was phenol/chloroform extracted, recovered by ethanol precipitation and redissolved in water.

Final size selection and removal of linkers

The DNA fragments were loaded onto a 60% Oligoprep gel (National Diagnostics) and separated using a Mini Protean II (Bio-Rad) vertical electrophoresis apparatus according to the manufacturer's recommendations. The gel was stained in 0.5µg/mL ethidium bromide for 30 mins and destained with water for 30 mins. Under UV illumination, the fragments were compared with a 50bp ladder (Gibco BRL) and those between 50bp and 350bp were excised using a scalpel blade. The DNA containing gel slice was macerated and solublised by adding 6 volumes of Oligoprep solubilising reagent. The DNA was purified from the resulting solution using Qiaex II kit (Qiagen) according to manufacturer's instructions and ethanol precipitation. The DNA was redissolved in water and quantitated by agarose gel electrophoresis and comparison with a DNA mass ladder (Gibco BRL).

Preparation of fUSE2 vector

fUSE2 replicative form (RF) was transformed into TG1 cells and plated onto LB agar containing 12.5µg/mL tetracycline. Transformants formed isolated colonies. A single colony was used to inoculate 50mL of LB broth containing 12.5µg/mL tetracycline. The culture was incubated overnight at 37°C in a shaker incubator. The culture was centrifuged at 6000g for 10 mins and the supernatant decanted. The fUSE2 RF was isolated using a Promega Wizard Midiprep kit according to the manufacturer's instructions. The fUSE2 RF DNA was *Bgl*II digested for 3 hours at 37°C, then more enzyme was added and incubated for another hour. The digested DNA was phenol/chloroform extracted, recovered by ethanol precipitation and redissolved in water. The DNA was dephosphorylated using calf intestinal alkaline phosphatase (Gibco BRL) at 0.01U/pmol 5' protruding ends in a reaction containing x10 CIAP buffer (Gibco BRL) and made up to volume with water. The reaction was incubated for 30 mins at 37°C, another 0.001U/pmol end of calf intestinal alkaline phosphatase was added and incubated for a further 30 mins. The reaction was stopped by adding 5µL of 250mM EDTA and incubating at 75°C for 10 mins. The reaction was purified using Qiagen PCR purification kit according to the manufacturer's instructions, ethanol precipitated and redissolved in water. An aliquot was tested by agarose gel electrophoresis for complete digestion and quantitated by comparison with a DNA mass ladder (Gibco BRL).

Ligation, electroporation and harvesting the library

The average fragment size was taken as 200bp and used to calculate the amount of DNA required to give the desired vector : insert ratios. Ligation reactions were set up at using a Stratagene ligation kit according to manufacturer's recommendations. The reactions setup were, a no-ligase control, a no-insert control and ligations with vector : insert ratios of 1:2, 1:3, 1:4 and 1:5. The reactions were incubated at 4°C overnight. Each reaction was purified using a Qiagen spin column, ethanol precipitated and redissolved in 2µL of water. 50µL of MC1061 electro-competent *E.coli* cells (Bio-Rad) was added to each sample, transferred to a 0.2cm electroporation cuvette (Bio-Rad) and electroporated at 12.5kV, 25µF, 200Ω using a Gene Pulse electroporator and pulse controller (Bio-Rad). The time constants achieved were 6.3mses in each case. An aliquot of each electroporation was sequentially 10-fold diluted and each dilution plated onto LB plates containing 12.5µg/mL tetracycline to determine the yield of each electroporation. The remaining cells were added to 250mL of LB broth containing 12.5µg/mL tetracycline and incubated overnight at 37°C. The culture was spun down at 6000g for 10mins. The supernatant was aspirated, reclarified by a further spin and the phage particles precipitated by the addition of 1/2 volume 30%(w/v)PEG/2.5M NaCl, mixed by inversion and allowed to stand at room temperature for 15 mins. The phage were pelleted by centrifuging at 10, 000g for 10 minutes and the supernatant carefully aspirated. The pellet was resuspended in 10mL of TE buffer and spun at 10,000rpm for 10 mins to pellet the residual cell debris. The supernatant was transferred to a fresh tube and the PEG precipitation repeated. The final pellet was resuspended in 2mL of TE buffer. This suspension represents the library of phage particles each carrying a displayed peptide. The final amplified library was titrated in TG1 cells on LB agar plates containing 12.5µg/mL tetracycline.

Characterisation of the library

10 discrete colonies were randomly selected from a titration plate, inoculated into 10mL of LB broth containing 12.5 µg/mL tetracycline and incubated overnight at 37°C. The cultures were spun at 6000g for 10 mins to pellet the cells. The supernatant was aspirated and discarded. The fUSE2 RF was isolated from the cells using a Wizard miniprep kit (Promega) according to the manufacturer's instructions. The purified DNA was used as the template in a PCR designed to amplify across the cloning site of the vector. The upstream primer was (cpIII-S) 5' GGTTGGTGCCTTCGTAGT 3' and the downstream primer (cpIII-T) 5' CCATGTACCGTAACACTG 3'. The components of the reaction

were as previously described and the thermal cycle was 25 cycles of 93°C for 60 secs, 45°C for 60 secs and 72°C for 90 secs. A 5µL aliquot of each reaction was electrophoresed through a 2.5% agarose gel containing 0.5µg/mL ethidium bromide and visualised under UV illumuination. This method was also used to determine the size of the inserts in a selection of colonies after biopan enrichment of the library.

Affinity selection of phage displayed peptides

Purified IgG (various) was diluted to 10µg/mL in 10mM sodium carbonate buffer, pH9.6 and 1mL dispensed in to a Maxisorp Immunotube (Nunc). The tube was capped and incubated at room temperature for 18 hours. It was washed three times with PBS containing 0.05% Tween 20 using a wash bottle. Excess wash fluid was removed using a disposable pipette. The tube was blocked by adding 1mL of PBS containing 2% dried milk powder and incubated at room temperature for 1 hour. Approximately 10^{10} library phage were added to a microfuge tube containing 1mL of PBS supplemented with 2% dried milk powder, 0.1%(v/v) E.coli/phage lysate (Stratagene) and 1µg/mL purified human IgG. The phage was incubated at 37°C for 1 hour. The blocking solution was aspirated from the coated tube and replaced with the diluted phage solution, mixed and incubated at 37°C for 3 hours with agitation. The tube was filled with PBS containing 0.05% Tween 20 using a wash bottle and thoroughly aspirated using a disposable pipette. This wash procedure was repeated a total of ten times. The bound phage was eluted by adding 1mL of 0.2M glycine/HCl buffer, pH2.2 for a maximum of 10 mins. The eluted phage were aspirated into a sterile universal, neutralised by adding 150µL of 1M Tris, pH9.1 and 200µL of TG1 plating bacteria was added. An aliquot of the eluted phage was retained for quantitation. The universal was agitated for 15 mins at 37°C to allow the phage to attach to the cells before 10mL of LB broth containing 12.5µg/mL tetracycline was added. The culture was agitated overnight at 37°C. It was spun at 6000g for 10 mins and the phage purified from the supernatant by two PEG precipitations. The eluted phage and the purified phage were titrated with TG1 cells on LB agar containing 12.5µg/mL tetracycline.

The biopanning procedure described above was repeated twice more, each time using the phage from the previous round as the starting material. In each successive round of biopanning the stringency of the wash protocol was increased. The second round was washed 15 times with PBS containing 0.1% Tween 20 and the third round was washed 20 times with PBS containing 0.5% Tween 20.

Analysis of biopan enriched phage

M13 immunoassay

Microplate wells were coated with either affinity purified antibodies specific for the major lipoproteins, and polyclonal anti-M13 antibody and purified normal human IgG. The antibodies to be immobilised were diluted to 5µg/mL in 0.01M carbonate buffer, pH9.6 and added at 100µL per well to Maxisorp 96-well microplates (Nunc), covered to prevent evaporation and incubated for 18 hours at room temperature. The wells were washed three times with PBS containing 0.05% Tween 20, then filled with a solution of 2% (w/v) dried milk powder and allowed to stand for 15 minutes. The plates were aspirated and any residual solution removed by inversion onto adsorbent paper towels. The plates were used immediately.

10^{10} phage were added to 100µL PBS, pH7.4 containing 0.05% Tween 20, 2% dried milk powder and 1µg/mL purified human IgG and allowed to stand for 1 hour. 100µL of the phage suspension was added to each well, the plate was sealed to prevent evaporation and incubated at 37°C for 2 hours. The plate was washed five times with PBS containing 0.05% Tween 20 and blotted dry on to adsorbent paper towels. Monoclonal anti-M13/horseradish peroxidase conjugate (Pharmacia 27-9421) was diluted 1:5000 in PBS, pH7.4 containing 0.05% Tween 20, 2% dried milk powder and 1µg/mL human IgG. 100µL of diluted conjugate was added to each well, the plate was sealed to prevent evaporation and incubated at 37°C for 2 hours. The plate was washed five times with PBS containing 0.05% Tween 20 and blotted dry on to adsorbent paper towels. Working strength substrate was prepared by adding 25µL of TMB chromogen to each 1mL of substrate solution. 100µL of working strength substrate solution was added to each well and incubated at room temperature for 30 mins. The enzyme reaction was stopped by adding 100µL of 0.25M H_2SO_4 to each well, mixed and the adsorbance read at 450 nm using a Multiskan plate reader (Labsystems).

DNA sequencing

Plasmid or RF DNA was isolated using Wizard purification kits (Promega) and ethanol precipitated. The vector / insert junctions of plasmid constructs were sequenced with standard T7 promoter (5' TAATACGACTCACTATA 3') and T7 terminator (5' CCGCTGAGCAATAACTAGC 3') primers. The pSurfscipt SK- plasmids were sequenced with a T3 promoter primer (5' ATTAACCCTCACTAAAGGGAA 3') and the fUSE2 RF DNA was sequences using the cpIII-seq

primer (5' CCCTCATAGTTAGCGTAACG 3'). DNA sequencing was performed by Alta Bioscience, University of Birmingham using an ABI Prism Model 377.

Chapter 3

Antibody responses to polypeptides of *T.pallidum*

Introduction

As previously discussed, the literature is unclear as to exactly which antigens are diagnostically significant. This is due to differences in nomenclature and techniques used by different researchers, which make the literature difficult to interpret. These initial experiments were performed to assist in the understanding of the literature and provide important first-hand observations of the research area. The results, in conjunction with the literature, should allow the diagnostically significant antigens to be identified for further study. The experiments also provide characterisation data on the antibody specificities present in each of the syphilitic serum specimens. This preparation provides useful baseline data for subsequent evaluations of recombinant proteins.

Results

The antibody response was investigated first by western blotting, using antigen from whole organisms separated on 10.5% SDS polyacrylamide gels and sera, diluted 1:100, from patients with different stages of syphilis. The resulting banding patterns were analysed using GelCompar™ (Applied Maths, Kortrijk) electrophoresis analysis software. Fig 1 shows that, early primary human sera react strongly and predominantly with polypeptides of 31, 35, 37 and 47 kDa. The most frequent response was to the 47 kDa polypeptide, virtually all sera reacting to a greater or lesser extent. Relatively few sera reacted, generally rather weakly, to polypeptides of 15 and 17 kDa. On the other hand late primary and secondary sera (Fig 2), while showing generally similar reactions as early primary to the higher molecular weight polypeptides, also show very strong reactions to a 44 kDa polypeptide which are now apparent in a slightly higher proportion of sera. The pattern is generally similar in latent (Fig 3) and old treated syphilis (Fig 4), with possibly a higher incidence in the latent group of reactions to the 44 kDa antigen. In re-infection sera (Fig 5) the same set of reactions is seen, and in addition reactivity to a diffuse antigen of around 24 kDa is common. Serum specimens from eighty-eight non-selected normal blood donors, seven patients with Lyme disease and twelve serologically confirmed biological false positives were tested and found to be non reactive with any identifiable polypeptides of *T.pallidum* (results not shown). Table 2 summarises these data, and it is apparent that no single pattern of reactivities characterises a particular stage of disease. It is also clear that the three main categories of sera liable to give false positive results by other serological tests do not give any reactions to the polypeptides specified above.

Figure 1. Western blot reaction patterns of serum specimens from 18 patients classified with early primary syphilis.

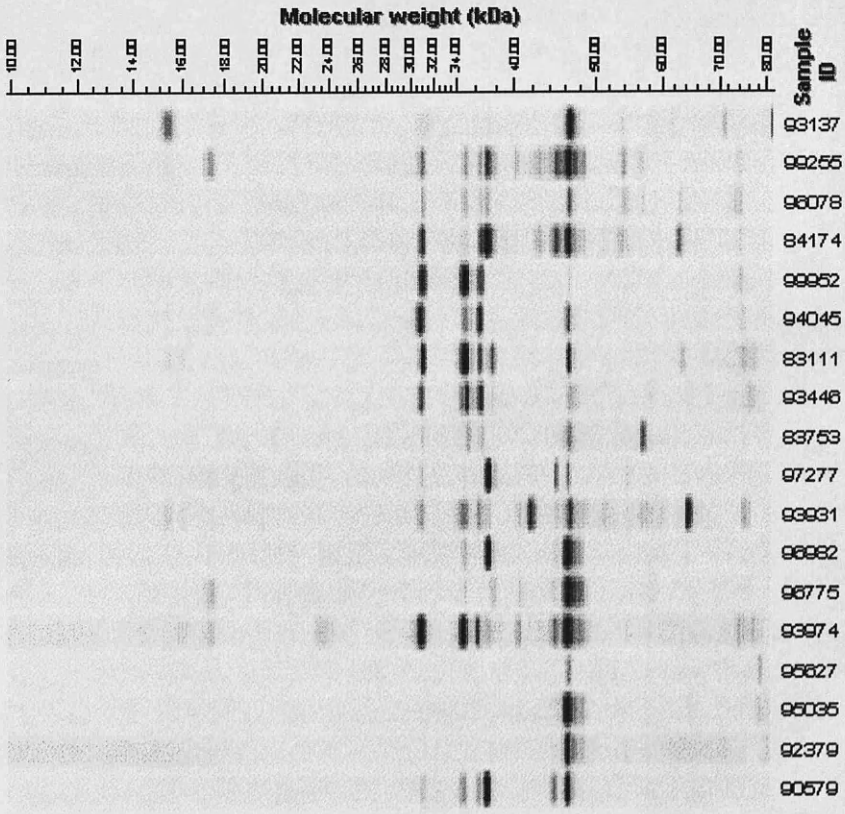


Figure 2. Western blot reaction patterns of serum specimens from 36 patients classified with late primary syphilis or secondary syphilis.

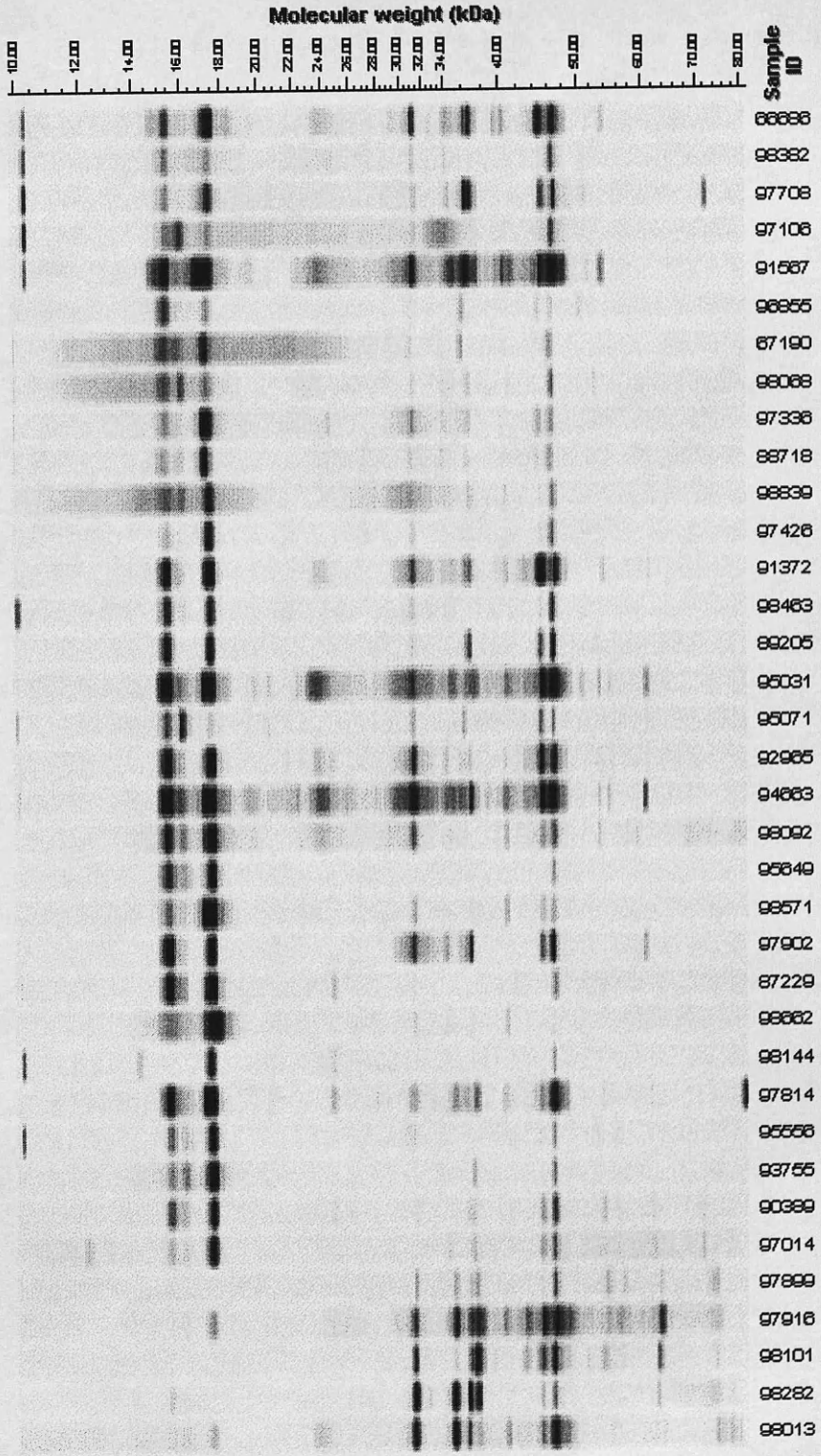


Figure 3. Western blot reaction patterns of serum specimens from 18 patients classified with latent syphilis.

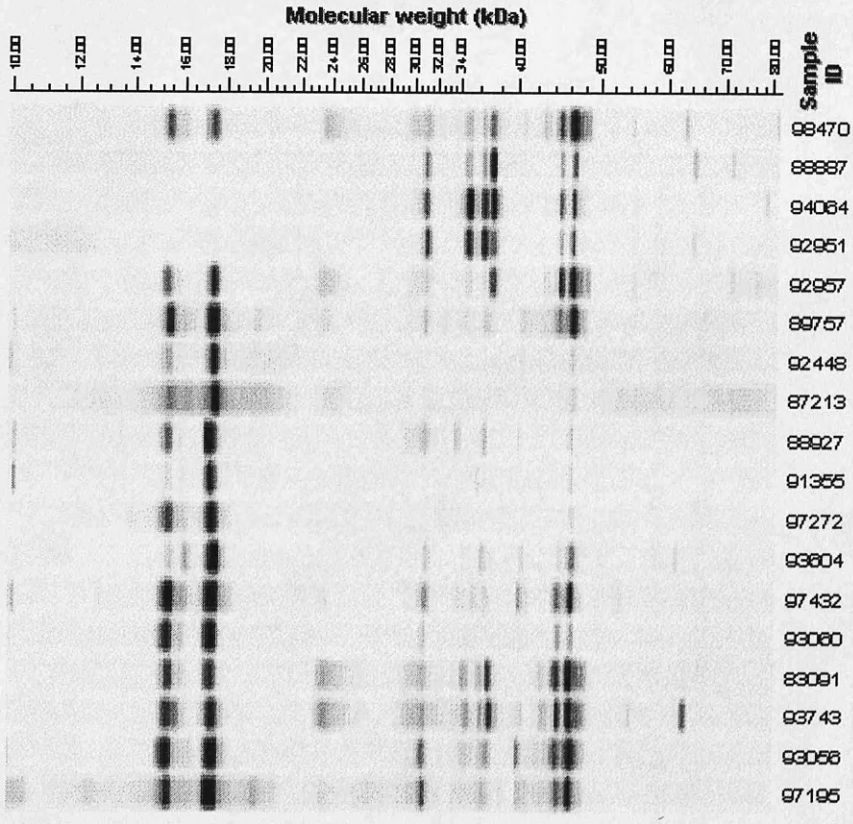


Figure 4. Western blot reaction patterns of serum specimens from 20 patients with successfully treated infections.

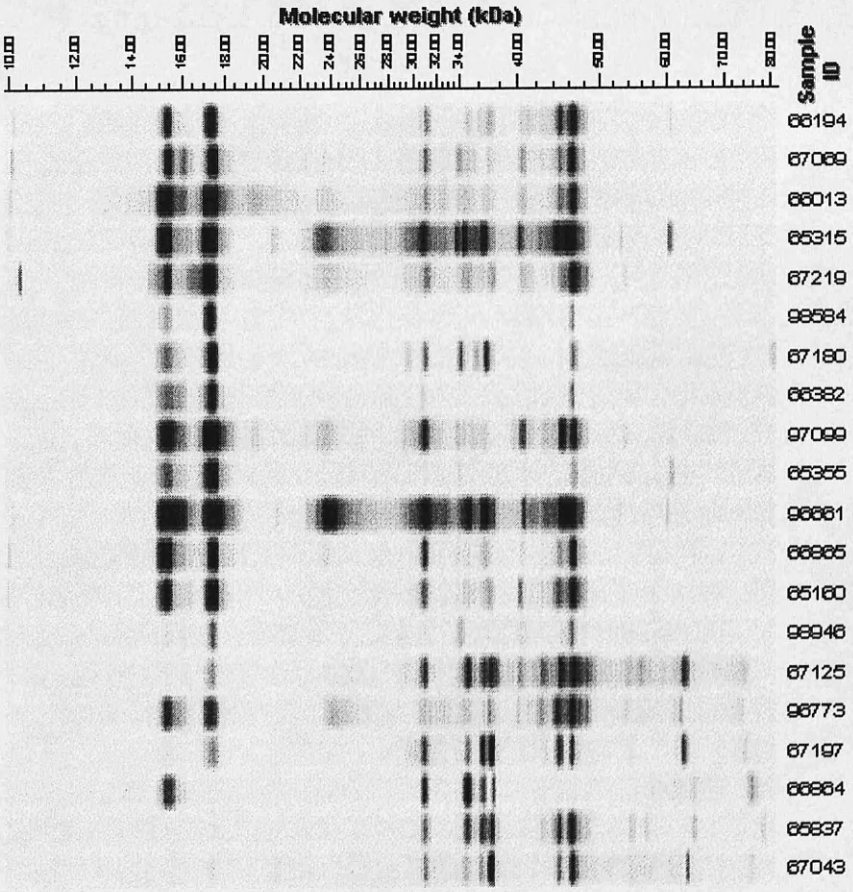


Figure 5. Western blot reaction patterns of serum specimens from 25 patients with well established infections or reinfections, characterised by high antibody titres.

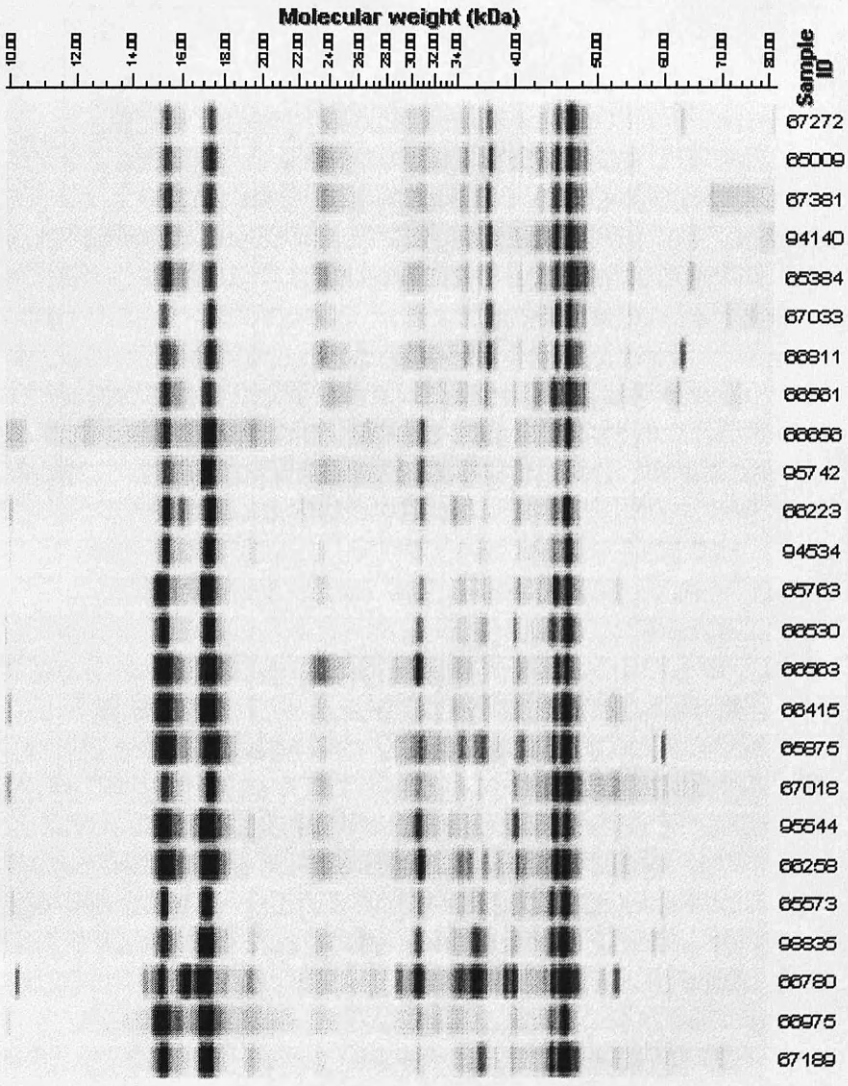


Table 2. Summary of the frequency of the different western blot reaction patterns of the major lipoproteins.

Reaction pattern				Disease stage of specimen							
47 kDa	44.5 kDa	17 kDa	15 kDa	Early Primary Syphilis	Late Primary/ Secondary syphilis	Latent Syphilis	Old treated infection	Reinfections	Normal Blood Donors	Lyme's Disease	Biological False Positives
+	+	+	+		21	10	10	25			
+	+	+	-	3	2	1	2				
+	+	-	-	5	1	2	2				
+	-	-	-	8	1		0				
+	-	+	-		1		1				
+	-	-	+	1			0				
+	-	+	+		8	5	4				
+	+	-	+	1	1						
-	-	-	+				1				
-	-	+	+		1						
-	-	-	-						88	7	12
Totals				18	36	18	20	25	88	7	12

Discussion

Antibody responses to *T.pallidum* proteins have been extensively studied in patients and infected animals by radioimmunoprecipitation (Alderete and Baseman, 1981; Baseman and Hayes, 1980; Moskophidis and Muller, 1984; Thormburg *et al.*, 1983) and western blotting (Baker-Zander *et al.*, 1985; Cockayne *et al.*, 1989; Dettori *et al.*, 1989; Hanff *et al.*, 1982; Hanff *et al.*, 1983; Lukehart *et al.*, 1982; Lukehart *et al.*, 1986; Norris and Sell, 1984; van Eijk *et al.*, 1982; Wicher *et al.*, 1989; Wicher *et al.*, 1986). However it is difficult to interpret much of this work because of inconsistent nomenclature of the proteins between different researchers. The work described here duplicates some of these previous studies. The intention was to use the information to reinterpret these studies in relation to current nomenclature. Generally these studies show that the early immune response is to several distinct antigens of between 30 kDa and 38 kDa, and to a highly antigenic polypeptide of around 45-48 kDa. As the disease progresses, so the number of reactive proteins

increases, with the most significant additional responses to proteins around 42 kDa, 12-14 kDa and 14-16 kDa. From the results described here these are probably the flagellin proteins, and TpN47, TpN44.5, TpN17 and TpN15, respectively. The flagellin proteins elicit strong antibody responses at most stages of the disease. However, the FlaB flagellins closely resemble those of other bacteria, so they are unlikely to be pathogen specific and hence to be of diagnostic significance. FlaA appears only to be present in certain spirochaetes, but is highly conserved in those sequences studied (Parales and Greenberg, 1991, 1993). This suggests that FlaA could be a diagnostically significant antigen, but it also has the potential to exhibit crossreactivity with antibodies produced against related organisms. The results did not reveal any significant antigenic polypeptides other than the four lipoproteins already extensively cited in the literature. Their strength and spectrum of reaction make them prime candidates for use in diagnostic tests. However, as western blots preferentially detect polypeptides which are well expressed in the organism, the results do not preclude the possibility that a poorly expressed polypeptide could be an important diagnostic antigen. Recombinant proteins are useful tools to examine this possibility as they can be expressed at much higher levels than native proteins, therefore allowing the diagnostic significance of an antigen to be determined independently of its natural level of expression.

The antigens of most interest as targets for the production of recombinant proteins are the strongly antigenic species; TpN47, TpN44.5, TpN17, and TpN15. Although TpN37 (FlaA) produces strong antibody responses, it is conserved in some spirochaetes, which does not make it a prime candidate for cloning. This limitation of TpN37 is further reinforced by the toxicity of the recombinant protein, experienced by researchers performing the initial cloning experiments, which led to difficulties in isolating and characterising the protein. This does not make FlaA an ideal initial target for cloning and expression, but it may be useful if the other recombinant proteins prove to be deficient in some way.

Chapter 4

Cloning the major lipoproteins of *T.pallidum*

Introduction

The main objective of these experiments was to clone the antigens identified in the previous experiments, and produce purified recombinant protein suitable for further serological studies. All the antigens described in this project have previously been cloned and sequenced by several researchers, as described in Chapter 1. The DNA sequences are readily available from Genebank or other similar databases, so the cloning experiments reported here are not new, but essential in order to provide recombinant protein for the subsequent work. The plasmid constructs provided for use in the experiments described here encoded the lipoproteins TpN44.5 and TpN17, and a previously poorly characterised lipoprotein, TpN24-28. TpN44.5 and TpN17 represent two of the major antigens identified from the previous experimental work. TpN24-28 was of interest because it appears as a diffuse, poorly reactive band in native antigen western blots due to its heterogeneous molecular weight. This property makes it difficult to observe and hence determine its diagnostic significance. The other prominent antigens identified in the previous experiments, TpN47 and TpN15, were cloned, expressed and purified as thioredoxin fusion proteins for further serological studies.

The use of recombinant proteins offers the opportunity to examine the antigenicity of the proteins independent of their natural levels of expression in the organism. Therefore, it is possible to identify weak responses not previously detected by the limited quantities of native antigen available in the organism.

TpN24-28

Results

The constructs pET50 and pET50REP, containing the genes encoding TpN24-28 and its modified form lacking the lipoprotein modification sequence, were provided by Ms. A. Ivic and Prof. C. W. Penn, University of Birmingham. They were expressed in *E.coli* BL21(DE3) cells and the results analysed by SDS polyacrylamide gel and western blot. Fig 6 and Fig 7 show the results of the expression of the pET50 and pET50REP constructs, respectively. The recombinant proteins are not obvious in the pre and post induction coomassie stained gel tracks (Lane 1 and 2) against the background of *E.coli* proteins, but the western blots clearly demonstrate their presence after induction (Lane 2). There is no detectable expression of either product before induction as shown by Lane 1 of the western blots. Expression of pET50REP produces a single distinct band, while pET50 produces two. Neither protein can be demonstrated in the culture media, even by western blot. After sonication of the harvested cells, the recombinant proteins are mostly found in the insoluble pelleted fraction, although a small quantity of material is released into the supernatant by sonication. Slightly more of the pET50REP product is seen in the soluble fraction than with the pET50 product, but overall expression levels of the two constructs seem similar. Lane 6 shows the material recovered after purification, using metal affinity chromatography to consist of just the recombinant proteins with no observable contaminating material. This purified protein was used for subsequent studies. The two recombinant products seem similar in terms of antigenicity and expression levels, so as the pET50 product resembles the native protein most closely, this material was used for the further studies.

Figure 6. Expression of pET50.

12% SDS polyacrylamide gel stained with coomassie blue and companion western blot developed with rabbit ant-*T.pallidum* antiserum. Lane M; pre-stained molecular weight markers (Mr are indicated). Lane 1; E.coli lysate pre induction. Lane 2; E.coli lysate 2 hours post induction. Lane 3; Culture medium 2 hours post induction. Lane 4; post sonication supernatant. Lane 5; post sonication pellet. Lane 6; purified recombinant protein.

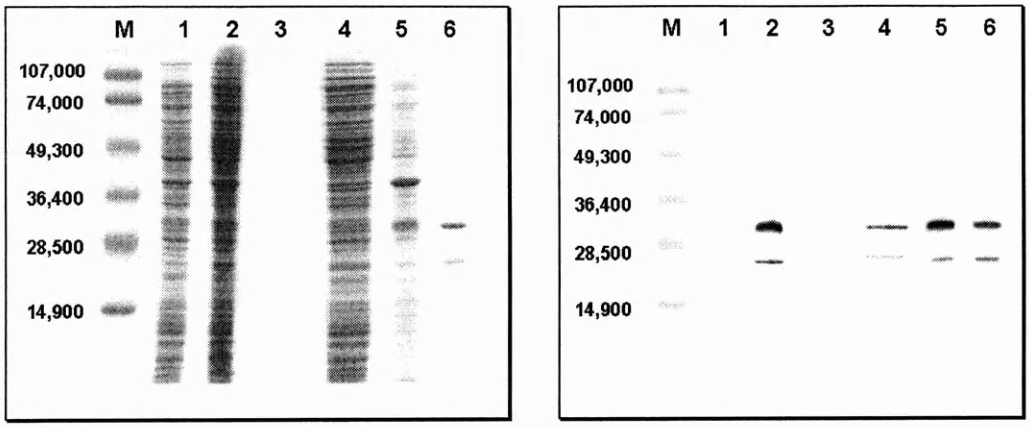
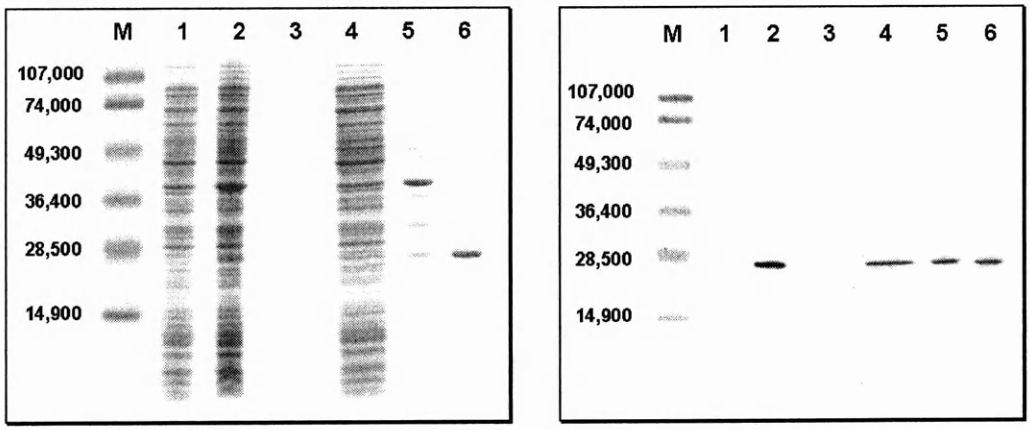


Figure 7. Expression of pET50REP.

12% SDS polyacrylamide gel stained with coomassie blue and companion western blot developed with rabbit ant-*T.pallidum* antiserum. Lane M; pre-stained molecular weight markers (Mr are indicated). Lane 1; E.coli lysate pre induction. Lane 2; E.coli lysate 2 hours post induction. Lane 3; Culture medium 2 hours post induction. Lane 4; post sonication supernatant. Lane 5; post sonication pellet. Lane 6; purified recombinant protein.



Discussion

The absence of the recombinant protein in the pre-induction samples is a result of the T7 promoter used in the pET vector series, which tightly controls expression of the cloned gene and minimises breakthrough expression of potentially toxic products. The pET50 construct carries the complete TpN24-28 gene sequence including the lipidation signal sequence and so should produce a fully lipid modified product. The presence of two polypeptides of different molecular weights suggests that processing of some kind is occurring. The finding that pET50REP, which lacks the lipid

modification signal sequence, produces a single protein species suggests that the most likely process occurring is lipid modification. However, the processing must be different from that which occurs naturally in *T.pallidum* as the product of pET50 does not show the heterogeneity in molecular weight seen in the native TpN24-28 protein. The majority of the recombinant protein is found in insoluble inclusion bodies within the cell and requires solubilisation with denaturing agents prior to purification. This insolubility probably results from a combination of the abnormally high level of expression of the recombinant proteins and the inability of *E.coli* to process the foreign material efficiently, which results in an accumulation of incorrectly folded protein. pET50REP was constructed to see if removal of the lipidation sequence improved the solubility and expression levels of the protein by making the product easier for the *E.coli* cell to process and therefore minimise the amount of insoluble material produced. This strategy seems to have failed as both constructs produced relatively similar quantities of product and there is no noticeable improvement in the solubility of the pET50 REP unmodified product.

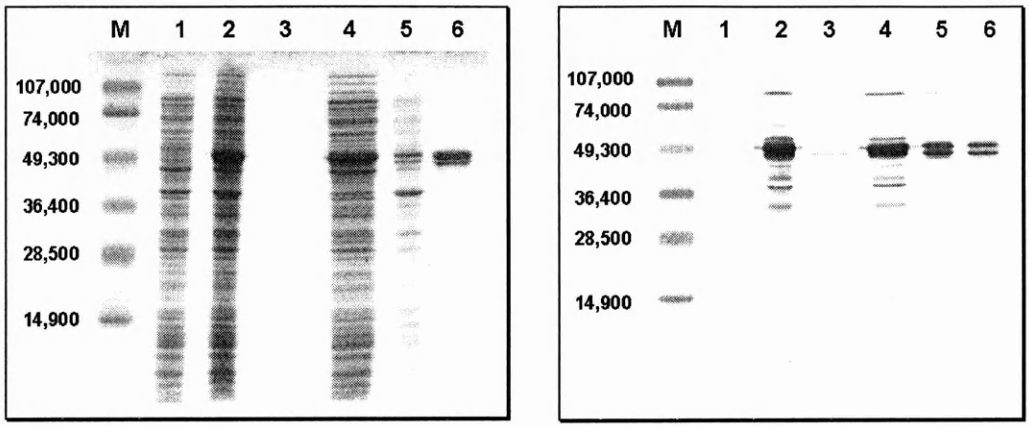
TpN44.5

Results

The construct pET10, containing the gene encoding TpN44.5 including the lipid modification sequence, was provided by Ms. A. Ivic and Prof. C. W. Penn, University of Birmingham. It was expressed in *E.coli* BL21(DE3) cells and the results analysed by SDS polyacrylamide gel and western blot (Fig 8). The recombinant products are visible in the coomassie stained, post induction sample (Lane 2) and absent prior to induction, which is confirmed by the western blot. The recombinant product appears as two major bands of closely related molecular weights and several other weaker bands at lower molecular weights, plus one band at a much higher molecular weight. No protein is detected in the culture medium (Lane 3) by coomassie staining, but western blotting reveals a single discrete band with a molecular weight similar to the lower of the two major bands in the post induction sample. After disruption of the cells by sonication, the recombinant products are partitioned fairly equally between the soluble (Lane 4) and insoluble fractions (Lane 5), with perhaps slightly more material present in a soluble form. Lane 6 shows that after purification using metal affinity chromatography the purified product contains approximately equal quantities of the two major polypeptide types with no other contaminating proteins detectable by coomassie staining.

Figure 8. Expression of pET10.

12% SDS polyacrylamide gel stained with coomassie blue and companion western blot developed with rabbit ant-*T.pallidum* antiserum. Lane M; pre-stained molecular weight markers (Mr are indicated). Lane 1; E.coli lysate pre induction. Lane 2; E.coli lysate 2 hours post induction. Lane 3; Culture medium 2 hours post induction. Lane 4; post sonication supernatant. Lane 5; post sonication pellet. Lane 6; purified recombinant protein.



Discussion

The pET10 construct contains the complete *tpn*44.5 gene including the lipidation signal sequence, so the expression product should be processed via the common acylation pathway. The presence of two polypeptide species suggests that some form of processing is occurring. The most likely explanation is that lipid modification is occurring, however the presence of large quantities of both species suggests that the process is far from complete. The level of expression seen is greater than that of the pET50/pET50REP constructs, which is probably related to how well the *E.coli* cells tolerate the foreign gene. The presence in the culture medium of a single protein band of a similar molecular weight to one of the two major expression products is an interesting finding. If it were due to being released from dead or damaged cells, then one would expect to find both of the major protein species occurring in similar relative proportions to those found in the post induction sample. The presence of only one of the species suggests that there maybe a selective process occurring, possibly an active export mechanism.

TpN17

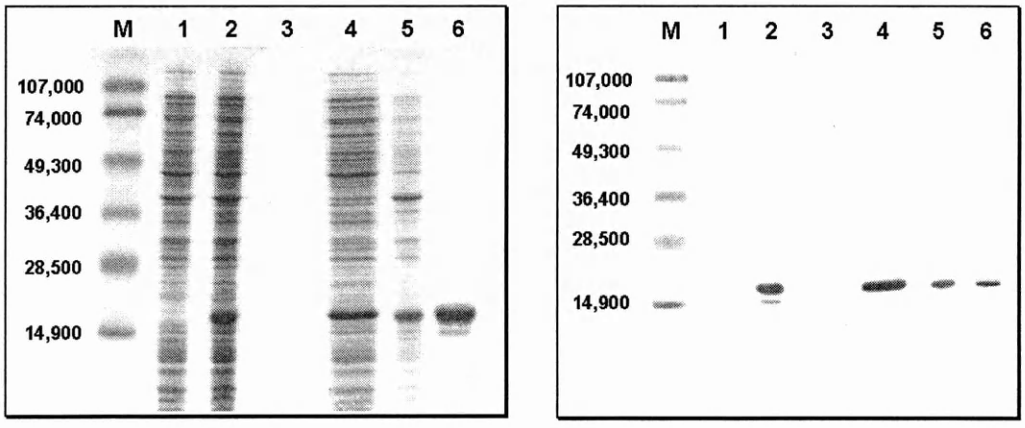
Results

The construct pET17, containing the gene encoding TpN17 including the lipid modification sequence, was provided by Ms. A. Ivic and Prof. C. W. Penn, University of Birmingham. It was

expressed in *E.coli* BL21(DE3) cells and the results analysed by SDS polyacrylamide gel and western blot (Fig 9). The results generally follow those previously described for the other constructs expressing lipoprotein sequences. Induction results in strong expression of the recombinant protein which can been seen in the coomassie stained post induction sample (Lane 2) as a large band that is not present prior to induction (Lane 1). This is confirmed by the western blot which shows the recombinant protein as a large, dark band with a much weaker lower molecular weight band below it. Lane 3 shows there is no detectable protein in the culture medium, even by western blot. After sonication of the harvested cells a large proportion of the recombinant protein is found in the soluble fraction (Lane 4). However, a significant fraction remains in an insoluble form (Lane 5). Lane 6 shows the recombinant protein after purification using metal affinity chromatography. The coomassie stained gel shows a weak band below the recombinant protein which is not detected in the western blot of the same sample.

Figure 9. Expression of pET17

12% SDS polyacrylamide gel stained with coomassie blue and companion western blot developed with rabbit ant-*T.pallidum* antiserum. Lane M; pre-stained molecular weight markers (Mr are indicated). Lane 1; E.coli lysate pre induction. Lane 2; E.coli lysate 2 hours post induction. Lane 3; Culture medium 2 hours post induction. Lane 4; post sonication supernatant. Lane 5; post sonication pellet. Lane 6; purified recombinant protein.



Discussion

This construct codes for the complete *tpn*17 gene including the lipidation signal sequence, therefore it should produce a lipid modified product. It produces good levels of expression and the product seems more soluble than the other lipoproteins described above. The recombinant protein appears

almost exclusively as a single entity, but there is some evidence of processing shown by the weak lower molecular weight band in the post induction sample. However, unlike the products of the other lipoprotein constructs, most of the pET17 product seems to occur as the higher molecular weight variant. The predominance of the higher molecular weight form and the superior expression levels may be due to better tolerance of the foreign gene by the *E.coli* cells resulting in more effective processing. The diffuse lower band seen in the purified protein sample that is not detected by western blot is unlikely to be unprocessed TpN17 due to its lack of reactivity with the antiserum. Two other possibilities are that it is either a contaminant protein or an artifact due to overloading of the gel track. No similar contaminant proteins have come through the purification method in the case of the other recombinant proteins, so this is unlikely to be the source. The most probable cause is smearing due to the high concentration of the protein loaded into that lane of the gel.

TpN15

Results

The construct pET60, containing the gene encoding TpN15 including the lipid modification sequence, was provided by Ms. A. Ivic and Prof. C. W. Penn, University of Birmingham. It was expressed in BL21(DE3) *E.coli* cells and the results analysed by SDS polyacrylamide gel and western blotting. Putative expression of the recombinant protein was only occasionally detected by western blotting after prolonged exposure to the blot development substrate. The bands were extremely faint which rendered them impossible to record by photography or scanning. The A_{600} of the culture was found to decrease after induction (data not shown).

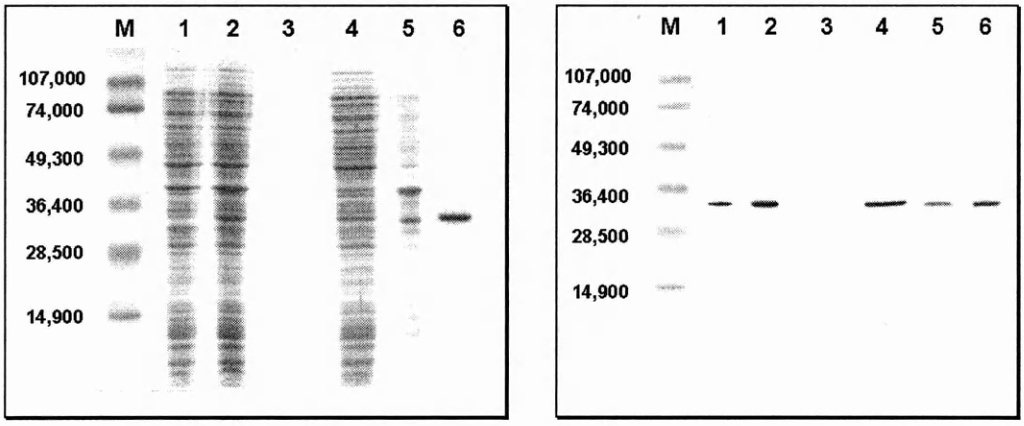
A construct, pET60REP, was prepared which coded for the *tpn*15 gene sequence except for the lipidation signal sequence. Sequencing of the vector / insert junctions confirmed the correct reading frame was maintained in the construct. It was expressed in BL21(DE3) *E.coli* cells exactly as the pET60 construct and the results were found to be identical.

Another construct, LIC15, was prepared which also coded for the *tpn*15 gene sequence without the lipidation signal sequence, but also resulting in a translational fusion to vector-coded thioredoxin. It was expressed in BL21(DE3) *E.coli* cells and the results analysed by SDS polyacrylamide gel and western blotting (Fig 10). After induction, the recombinant protein can be seen against the

background of E.coli proteins by coomassie staining (Lane 2). The western blot shows that the recombinant protein is slightly expressed before induction (Lane 1). Not surprisingly, no protein is detected in the culture medium. After sonication, most of the recombinant protein is found in the soluble fraction (Lane 4) with some remaining associated with the insoluble material (Lane 5). Lane 6 shows that, after metal affinity chromatography, the purified material contains a single polypeptide band.

Figure 10. Expression of LIC15.

12% SDS polyacrylamide gel stained with coomassie blue and companion western blot developed with rabbit ant-*T.pallidum* antiserum. Lane M; pre-stained molecular weight markers (Mr are indicated). Lane 1; E.coli lysate pre induction. Lane 2; E.coli lysate 2 hours post induction. Lane 3; Culture medium 2 hours post induction. Lane 4; post sonication supernatant. Lane 5; post sonication pellet. Lane 6; purified recombinant protein.



Discussion

The pET60 construct codes for the complete *tpn*15 gene sequence including the lipidation signal sequence which means the product should be lipid modified by the common acylation pathway. The extremely poor expression and apparent cell lysis after induction suggests that the recombinant protein produced is poorly tolerated and possibly toxic to *E.coli.* A construct expressing the *tpn*15 gene sequence without the lipidation signal sequence, pET60 REP, was constructed in an attempt to increase the tolerance of *E.coli* to the recombinant protein. The hypothesis was that an unmodified protein might be more easily processed and the lack of the lipid might hinder any functional activity the protein might have. As the outcome was virtually identical to the mature lipidated protein one can only conclude that if the lack of expression is due to toxicity then it is a function of the amino acid sequence and not dependent on the lipid content of the protein. This concept was taken further with the construction of LIC15. The sequence of *tpn*15 was cloned

without the lipid modification sequence and expressed as a fusion with a vector encoded protein, thioredoxin. This should not only prevent any residual functional activity TpN15 may exhibit but also improve the solubility of the recombinant protein. Recombinant proteins which are normally produced in an insoluble form in the cytoplasm of *E.coli* have been shown to become more soluble when fused with thioredoxin (LaVallie *et al.*, 1993). This strategy seems to be successful as it produces good expression of a recombinant protein. However, the presence of the recombinant protein prior to induction suggests that the control of the expression is not as tight as in the pET series of constructs. This is probably a function of the vector used rather than due to the cloned gene it contains. The presence of the fusion protein may interfere with the antigenicity of the TpN15 polypeptide by altering the secondary structure of the protein thereby eliminating or masking key epitopes. The modification of the protein by fusion does not seem to affect the antigenicity as the recombinant product reacts strongly with the anti-*T.pallidum* antiserum tested. Whether or not the fusion enhances the solubility of the recombinant protein is difficult to judge. Although the majority of the recombinant can be recovered in the soluble fraction, the complete failure to generate a non-fusion construct for comparison means no firm conclusions can be drawn. But the very fact that this construct expresses useful levels of protein, whereas the pET constructs produced nothing, suggests that the fusion is exerting a significant influence on the TpN15 sequence product.

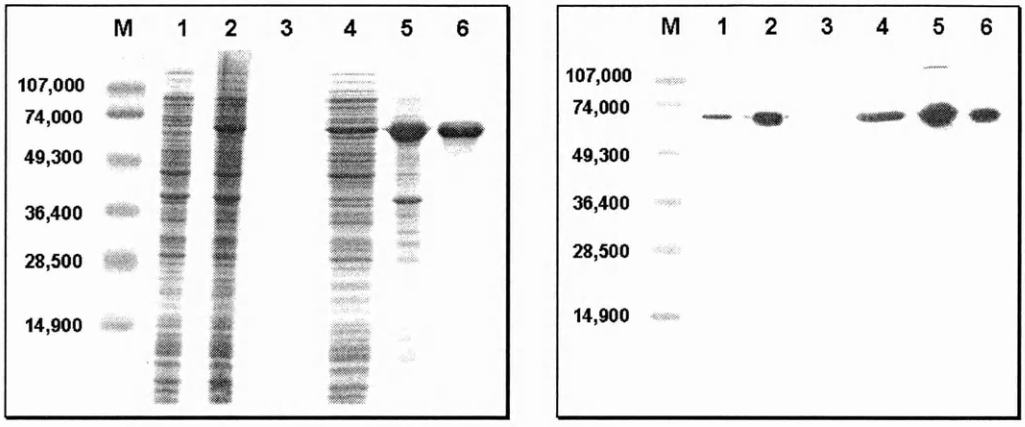
TpN47

Results

The construct LIC47, containing the *tpn*47 gene without the lipidation signal sequence, but also resulting in translational fusion to vector-coded thioredoxin, was prepared and expressed in BL21(DE3) *E.coli* cells. The results were analysed by SDS polyacrylamide gel and western blot (Fig 11). The most obvious point to note is the high levels of expression seen with this recombinant protein compared to most of the others described in this chapter. It can be clearly seen as a large band on the polyacrylamide gel after induction (Lane 2), which appears absent from the pre-induction sample (Lane 1). However, the western blot reveals that a small amount of expression is actually occurring prior to induction. Most of the material separates to the insoluble fraction (Lane 5) and purifies to a single entity after metal affinity chromatography (Lane 6).

Figure 11. Expression of LIC47.

12% SDS polyacrylamide gel stained with coomassie blue and companion western blot developed with rabbit ant-*T.pallidum* antiserum. Lane M; pre-stained molecular weight markers (Mr are indicated). Lane 1; E.coli lysate pre induction. Lane 2; E.coli lysate 2 hours post induction. Lane 3; Culture medium 2 hours post induction. Lane 4; post sonication supernatant. Lane 5; post sonication pellet. Lane 6; purified recombinant protein.



Discussion

As no transformants were produced after numerous attempts to clone *tpn*47 into a pET21b, so the strategy used to clone *tpn*15 was adopted and proved successful. The expression levels achieved by this construct greatly exceed those seen with the other constructs described in this chapter. Obviously, expression of the thioredoxin-TpN47 fusion protein is well tolerated by *E.coli* resulting in very high expression levels being seen after induction. Compared to the amount of protein produced after induction, the small amount seen prior to induction is minimal and may just be a product of the high expression levels achieved. The breadth of the bands on the gel and western blot is a result of the amount of protein present causing distortion of the gel track and not due to heterogeneity in the molecular weight. When smaller amounts of protein are electrophoresed the recombinant protein forms narrow discrete bands. The presence of the fusion protein does not appear to alter the antigenicity of the TpN47 portion of the recombinant protein as strong reactions are seen with the anti-*T.pallidum* antiserum.

Summary

TpN44.5 and TpN17 were successfully expressed from the full lipoprotein sequences including the lipid modification sequence. The plasmids seem to be well tolerated by the *E.coli* cells and expression levels were adequate to produce reasonable quantities protein that was readily purified

from inclusion bodies. There is evidence that the proteins are lipid-modified and seem to be antigenic judged by their reactions with anti-*T.pallidum* antiserum.

TpN24-28 was expressed from two constructs, as the full lipoprotein sequence and as the mature protein excluding the lipid modification sequence. The full lipoprotein seems to be processed by the *E.coli* cells, which is shown by the two bands on SDS-PAGE. This is further reinforced by the single band produced by the truncated gene, suggesting that removal of the lipid modification sequence prevents processing occurring. The plasmids are tolerated by the *E.coli* cells but expression levels are lower than those seen with the previous two constructs. The expression levels of the two TpN24-28 variants seem similar, so elimination of processing by the deletion of the lipid modification signal sequence does not enhance expression. The fully lipid-modified version was purified and used for the subsequent studies as it was thought most closely to resemble the native molecule.

TpN15 proved highly problematic to clone and express. Constructs expressing the gene, both with and without the lipid modification sequence, were poorly tolerated by the *E.coli* cells and probably toxic. Induction of the gene resulted in a decrease in the number of cells in the culture and poor expression of the recombinant protein. TpN15 was finally successfully cloned and expressed as the mature protein sequence, minus the lipid modification sequence, fused to vector-coded thioredoxin. This plasmid was well tolerated by the *E.coli*, resulting in high expression of the recombinant protein which was easily purified by metal affinity chromatography. The fusion protein did not appear to affect the antigenicity of the recombinant protein as judged by its reaction with anti-*T.pallidum* antiserum.

The TpN47 was successfully cloned and expressed using an identical strategy to that used for TpN15. The construct was very well tolerated by the *E.coli* cells and resulted in exceptionally high levels of expression. The recombinant protein was purified using metal affinity chromatography and its antigenicity appeared to be unaffected by the presence of the fusion protein, judged by its reaction with anti-*T.pallidum* antiserum.

All the purified proteins were stored frozen and used for further serological studies.

Chapter 5

Antigenicity of TpN24-28

Introduction

TpN24-28 is a previously poorly characterised lipoprotein that shows molecular weight heterogeneity by SDS-PAGE. This produces a diffuse, weakly reactive band in native antigen western blots. An antibody response directed towards it is only observed in a proportion of patients with well established infections. This could be due to its diffuse nature making observation difficult, or to the protein being poorly immunogenic. The objective of this experiment was to evaluate the frequency and strength of the response to TpN24-28 using recombinant protein. The recombinant TpN24-28 can be tested at higher concentrations than is possible using native antigen and therefore should determine its true antigenicity independent of natural levels of expression. The testing was performed by western blot, even though the material was purified, because it offers a qualitative evaluation that is independent of purity. Reactions to TpN24-28 can be easily distinguished from those directed towards contaminating proteins in the antigen preparation. Recombinant TpN44.5 and TpN17 were used for comparison as antibody responses are commonly seen to these proteins, especially in established infections.

Results

The reactivity of syphilitic and non-syphilitic serum specimens with the recombinant TpN24-28 was investigated by western blotting. TpN44.5 and TpN17 were also tested for comparison and to act as controls. All gels are 12% acrylamide covering molecular weights from >200 kDa to <10 kDa, and are shown with high molecular weight to the right. Fig 12 shows that all 38 serum specimens from patients with syphilis are clearly reactive with TpN44.5 and all 20 normal antenatal specimens are non-reactive. A similar picture is seen in Fig 13 which shows the reactivity of the same specimens to TpN17. Again, all syphilitic sera are clearly reactive and the normal specimens are non-reactive with TpN17. A different picture is seen in Fig 14 where the same specimens are tested against TpN24-28. All the normal antenatal specimens are non-reactive with TpN24-28 and only 13 of the 38 syphilitic serum specimens are strongly reactive, two are weak and a further one very faint. There are additional reactivities seen at higher molecular weights, to the right of the figures, which are present in a large number of specimens both syphilitic and non-syphilitic.

Figure 12. Reactivity of recombinant TpN44.5 with syphilitic and non-syphilitic serum specimens.

Western blot reaction patterns of serum specimens of 38 serologically reactive patients with established syphilis infections, 20 non-selected antenatal patients negative for syphilis, and two controls. The positive and negative controls are a high titre syphilitic serum and a pool of sera from non-reactive blood donors.

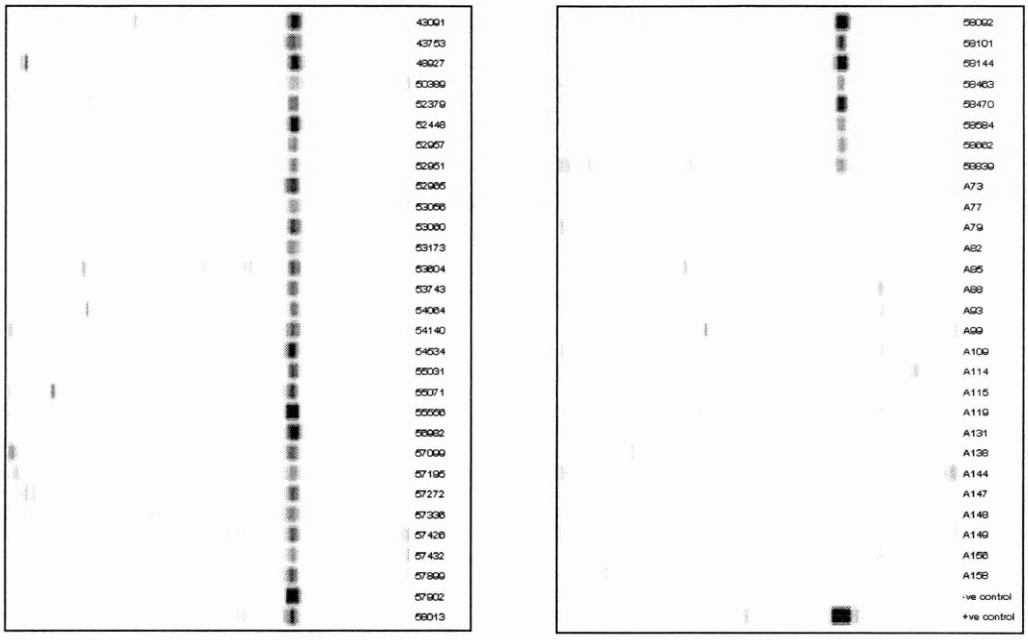


Figure 13. Reactivity of recombinant TpN17 with syphilitic and non-syphilitic serum specimens.

Western blot reaction patterns of serum specimens of 38 serologically reactive patients with established syphilis infections, 20 non-selected antenatal patients negative for syphilis, and two controls. The positive and negative controls are a high titre syphilitic serum and a pool of sera from non-reactive blood donors.

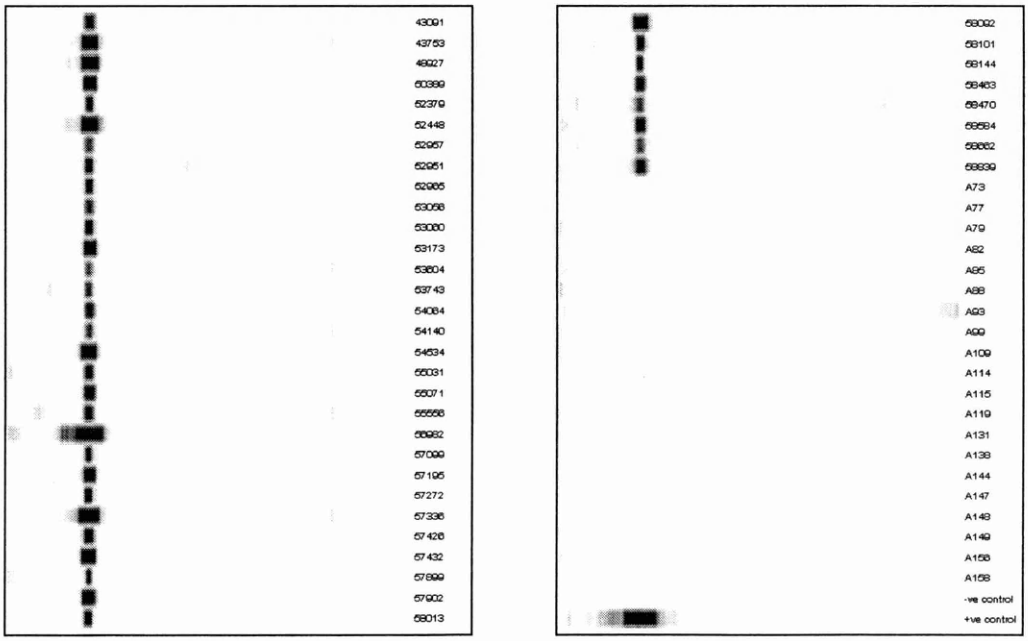
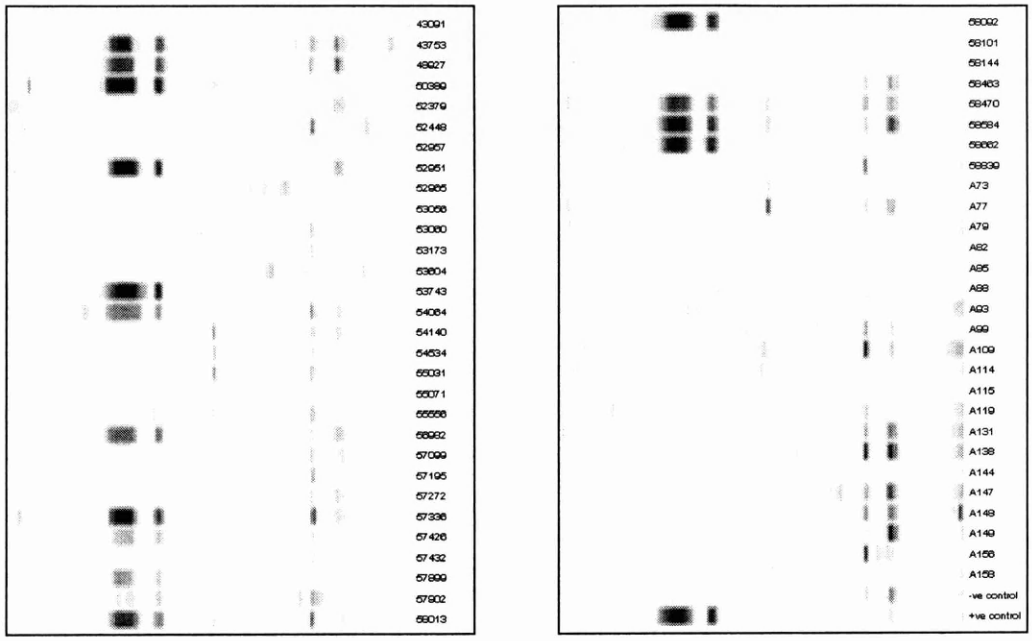


Figure 14. Reactivity of recombinant TpN24-28 with syphilitic and non-syphilitic serum specimens.

Western blot reaction patterns of serum specimens of 38 serologically reactive patients with established syphilis infections, 20 non-selected antenatal patients negative for syphilis, and two controls. The positive and negative controls are a high titre syphilitic serum and a pool of sera from non-reactive blood donors.



Discussion

Western blot results are affected by the amount of each antigen present in the cell and they tend to favour the detection of antibodies against abundant proteins. TpN24-28 is thought to be pathogen specific, but produces a diffuse weak reaction in western blots due to the variation in molecular weight and low level of expression in the organism. Putative reactivity to TpN24-28 can be seen in some specimens from patients with well established disease in the western blot studies described in Chapter 3. The infrequency of the response may be due to the low level of expression and the heterogeneous nature of the antigen making detection difficult, or the response to TpN24-28 may develop late in the disease. It is difficult to assess the diagnostic significance of this antigen using whole treponemes. However, recombinant proteins can be expressed at a much higher level and so are not limited in this way. The product of pET50 was used for this study as it should be lipid modified and therefore is the material most closely resembling native TpN24-28.

Although the material was purified, western blotting was still used as it provides qualitative information about the nature of the reactions, thereby allowing the differentiation between true reactivity and reactions with contaminating proteins. It is clear from the data that in contrast to the

reactions with TpN44.5 and TpN17, reactivity to TpN24-28 is far more erratic. A large number of the specimens tested showed reactions with high molecular weight species that were most probably *E.coli* proteins not removed by the purification process. If a quantitative method, such as enzyme immunoassay, had been used these reactions could lead to the conclusion that TpN24-28 was more reactive than it actually is. TpN24-28 was non reactive with all the normal antenatal specimens which, although a rather limited evaluation, hints at the specific nature of the protein. The lack of reactivity with all the syphilitic specimens means that it would not be diagnostically significant by itself, but it could be useful in conjunction with other proteins. However, as all the specimens that reacted with TpN24-28 also reacted strongly with TpN44.5 and TpN17, its inclusion in a mixture would be unlikely to add any extra reactivity. For this reason TpN24-28 was not investigated further during this study. The attention was therefore focused on the four disease specific lipoproteins that were thought to show strong reactivity in most stages of the disease, TpN47, TpN44.5, TpN17 and TpN15.

Summary

This preliminary evaluation suggests that TpN24-28 seems to be pathogen specific as judged by the lack of response with any of the antenatal specimens tested. In order to confirm this many more specimens from patients with no history of syphilis would have to be tested, as well as specimens from potentially cross reactive sources. However, the sporadic nature of the antibody response to it, compared to the other lipoproteins tested, means that it is not reactive frequently enough to provide any additional antigenicity over more universally reactive antigens. Therefore, TpN24-28 was not investigated further during the study reported here.

Chapter 6

Immunoblotting of recombinant proteins

Introduction

The antibody responses to the recombinant proteins of a range syphilitic serum specimens were evaluated by western blotting. This particular technique was applied because it allows a qualitative assessment of the responses which is not reliant on the purity of the antigen preparations. The technique is also fairly robust and potentially highly sensitive, mainly due to the large quantities of antigen that can be immobilised on the nitrocellulose membrane. The objective was to determine the frequency and strength of the antibody responses to the recombinant proteins at all stages of the disease, and compare the results with the responses of the same sera to native antigen. This would allow comparison of the specificity of the recombinant proteins with native antigen to be made and might highlight any effects of the fusion proteins on antigenicity. The results also provided baseline data for comparison with any subsequent experimental results, and if necessary allow resolution of any discrepancies.

Results

The four recombinant proteins, TpN47, TpN44.5, TpN17 and TpN15, were tested by western blotting against a range of serum specimens from patients with different stages of syphilis infection. All gels were 12% acrylamide and covered molecular weights from >200 kDa to <10 kDa, and are shown with high molecular weight to the right.

TpN47

All the early primary (Fig 15), late primary / secondary (Fig 16) and latent syphilis specimens (Fig 17) were reactive. Generally, the reactions were strong and clear, with only the occasional weaker reaction. Fig 18 shows that all, except one, of the specimens from successfully treated cases were also reactive.

Figure 15. Reactivity of recombinant TpN47 in early primary syphilis.

Western blot reaction patterns of serum specimens from 18 patients classified with early primary syphilis tested against recombinant TpN47.

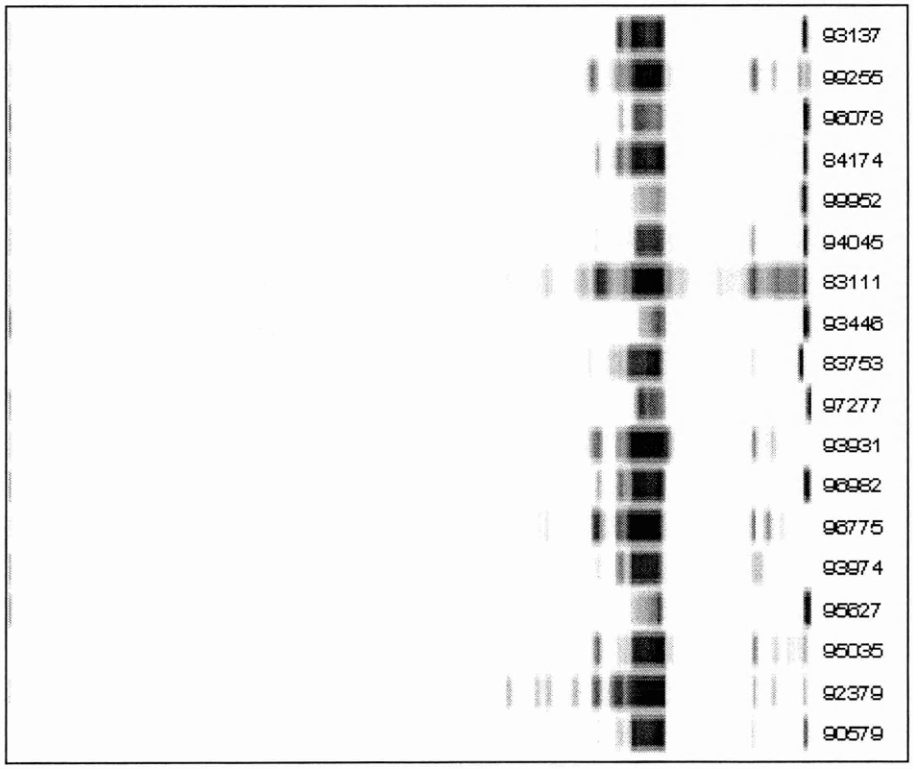


Figure 16. Reactivity of recombinant TpN47 in late primary / secondary syphilis.

Western blot reaction patterns of serum specimens from 36 patients classified with late primary syphilis or secondary syphilis tested against recombinant TpN47.

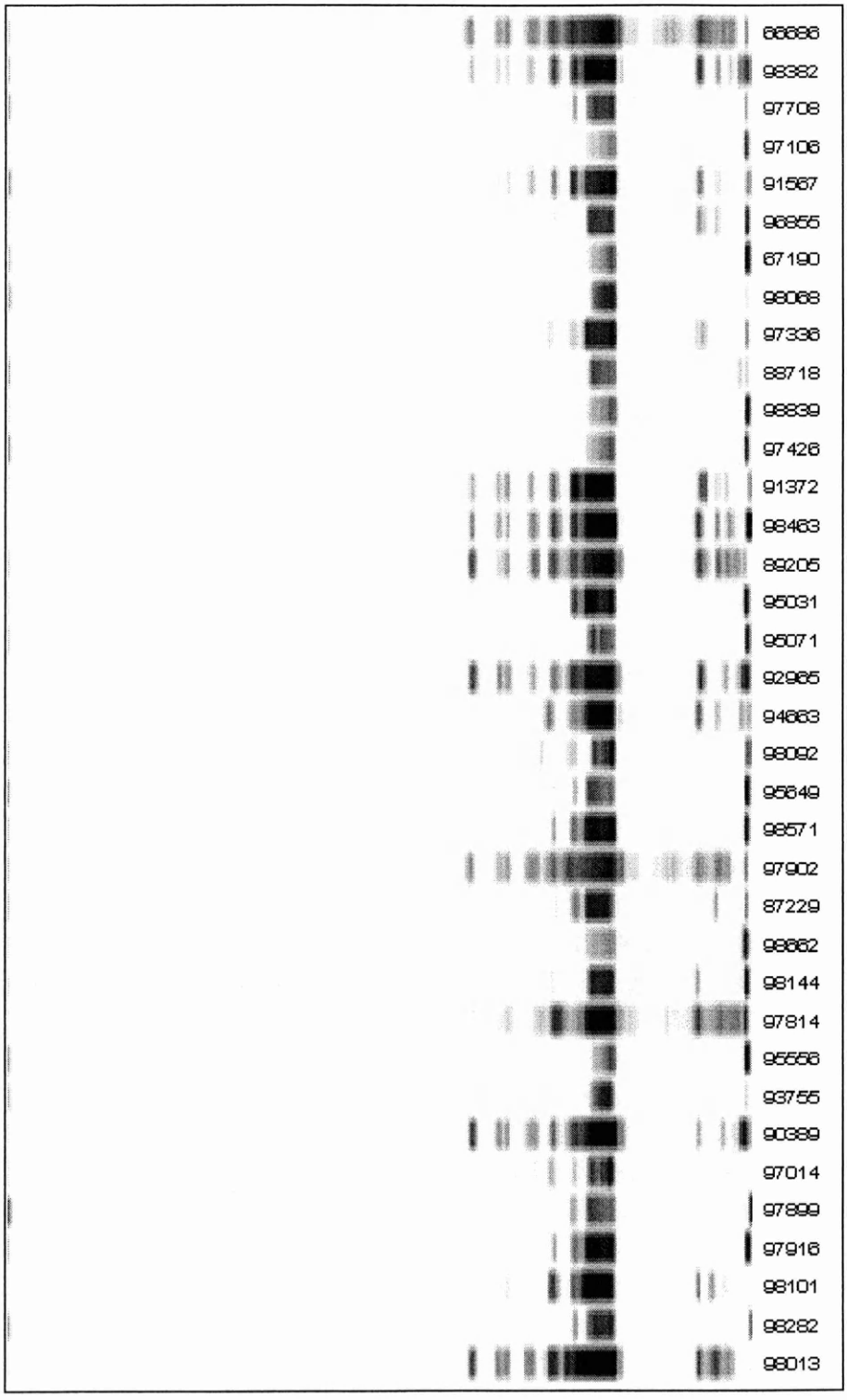


Figure 17. Reactivity of recombinant TpN47 in latent syphilis.

Western blot reaction patterns of serum specimens from 18 patients classified with latent syphilis tested against recombinant TpN47.

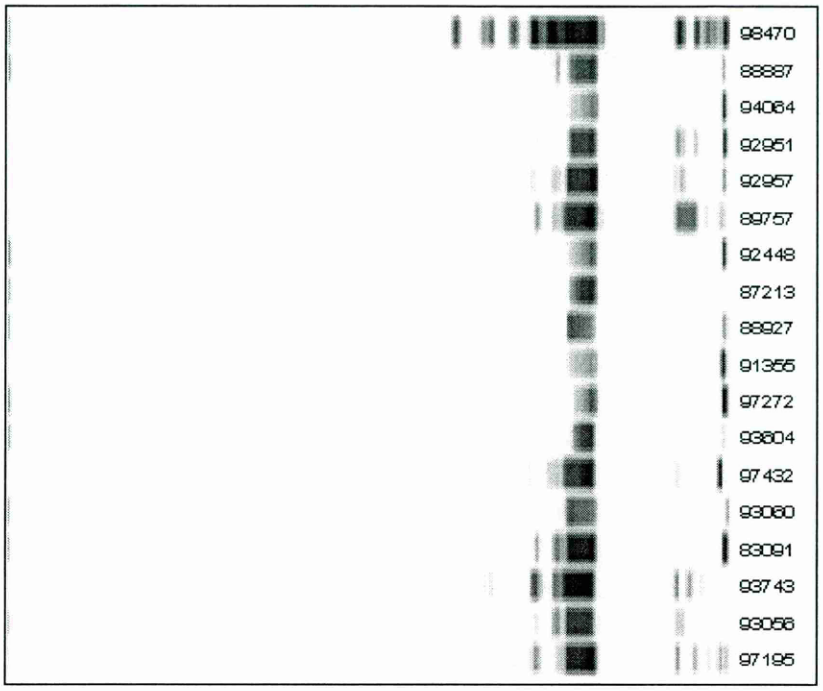
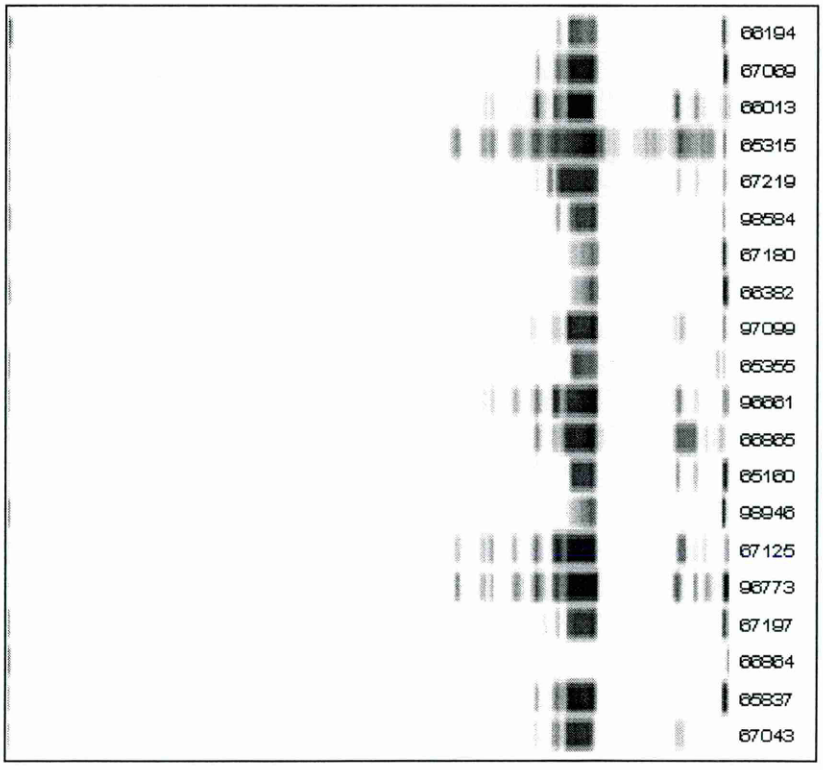


Figure 18. Reactivity of recombinant TpN47 in successfully treated cases.

Western blot reaction patterns of serum specimens from 20 patients with successfully treated infections tested against recombinant TpN47.



TpN44.5

Generally, the reactions with TpN44.5 are weaker and more variable in strength than is seen with TpN47. Fig 19 shows that most of the early primary specimens react weakly, however only three specimens are actually completely non-reactive. A similar picture can be seen in Fig 20 with the late primary / secondary specimens where the reactions are predominantly fairly weak, but with a greater proportion clearly reactive, and only two specimens completely non-reactive. The latent syphilis specimens (Fig 21) divide fairly evenly with nine reacting strongly and seven weakly. The remaining two specimens are non-reactive. Most of the treated cases (Fig 22) are strongly reactive with only one specimen non-reactive.

Figure 19. Reactivity of recombinant TpN44.5 in early primary syphilis.

Western blot reaction patterns of serum specimens from 18 patients classified with early primary syphilis tested against recombinant TpN44.5.

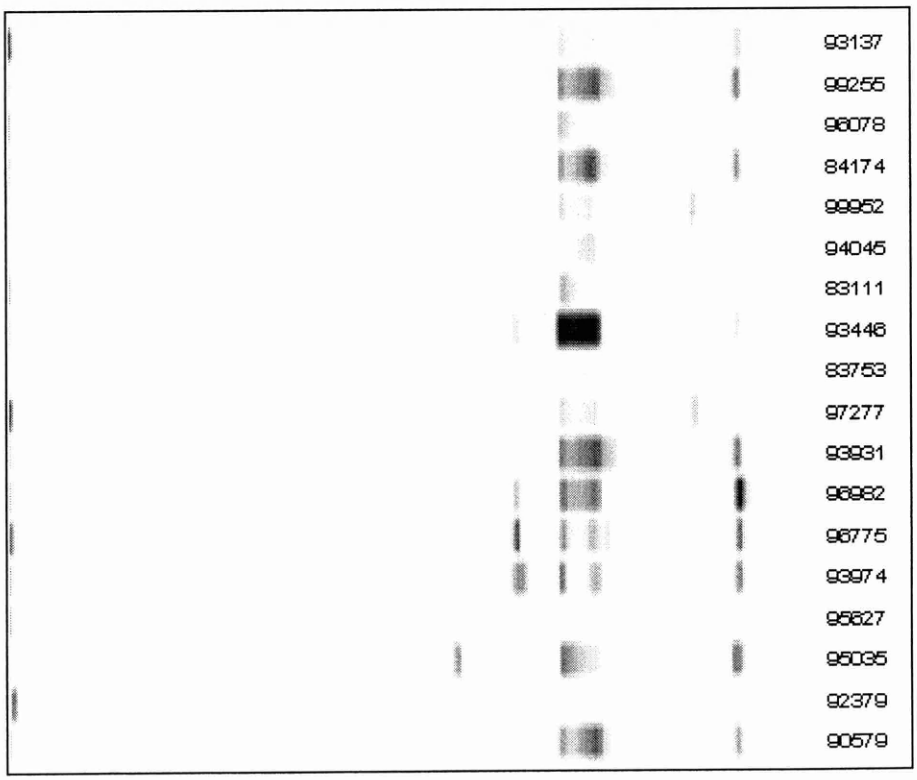


Figure 20. Reactivity of recombinant TpN44.5 in late primary / secondary syphilis.

Western blot reaction patterns of serum specimens from 36 patients classified with late primary syphilis or secondary syphilis tested against recombinant TpN44.5.

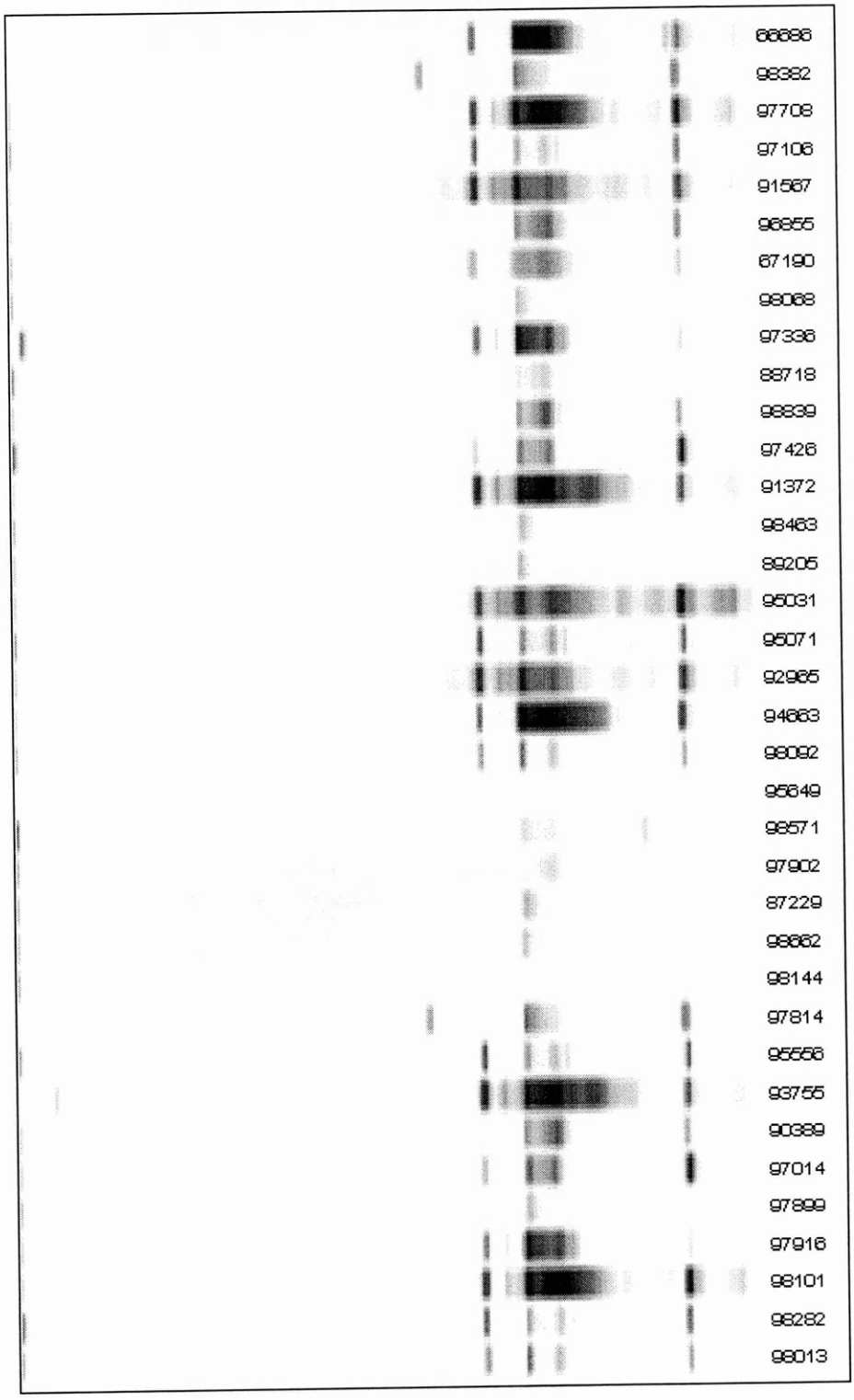


Figure 21. Reactivity of recombinant TpN44.5 in latent syphilis.

Western blot reaction patterns of serum specimens from 18 patients classified with latent syphilis tested against recombinant TpN44.5.

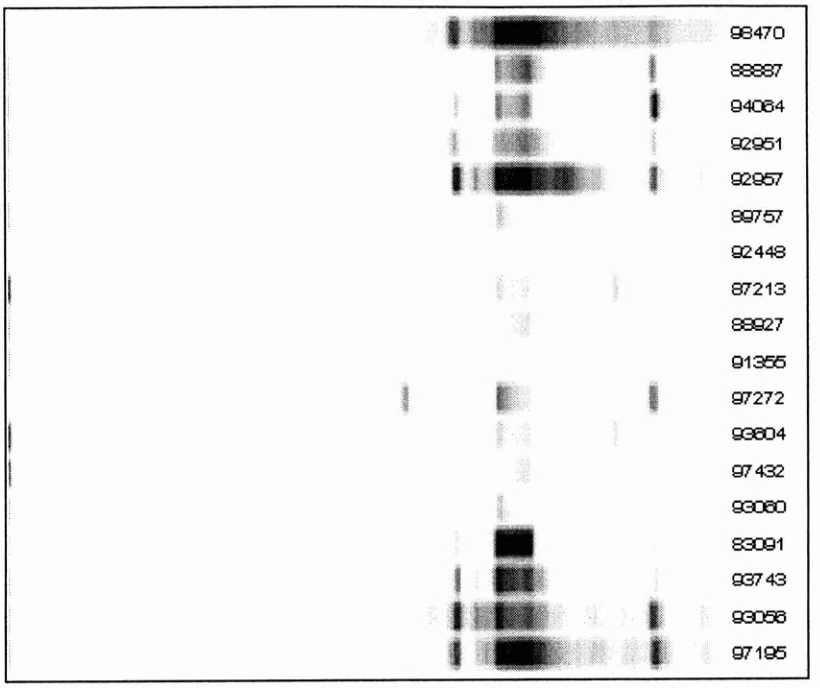
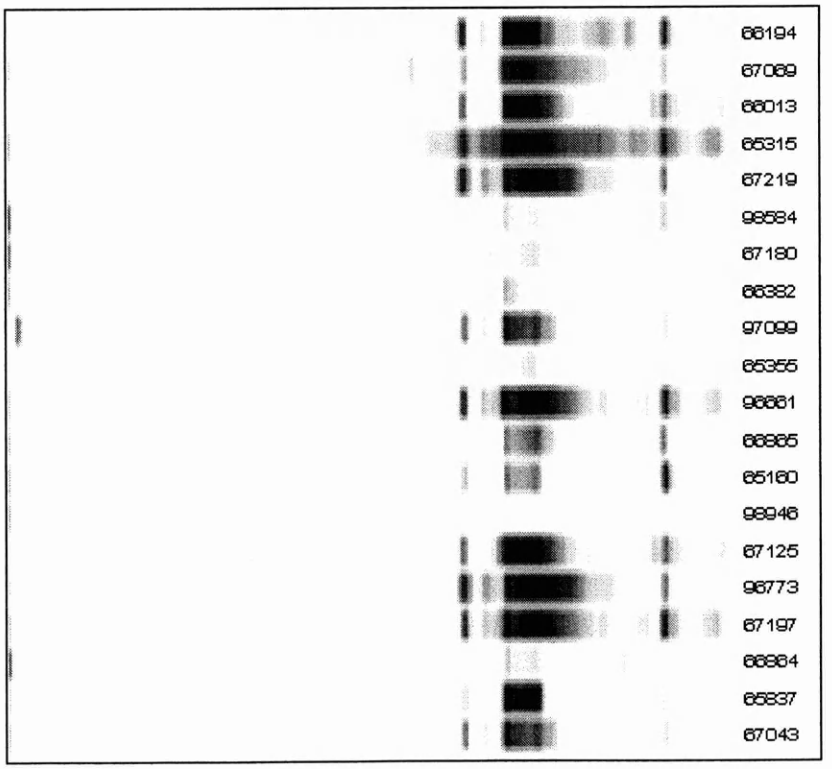


Figure 22. Reactivity of recombinant TpN44.5 in successfully treated cases.

Western blot reaction patterns of serum specimens from 20 patients with successfully reacted infections tested against recombinant TpN44.5.



TpN17

The reactions with TpN17 are more consistent in strength than those seen with the previously described proteins and the overall picture is different. Fig 23 shows that the early primary specimens are generally non-reactive, with only six specimens giving weak responses. The late primary / secondary specimens (Fig 24) show the opposite picture, where most are clearly reactive with very few weak responses and two non-reactive specimens. This picture continues for the specimens from patients with latent syphilis (Fig 25) and the successfully treated cases (Fig 26) which are mostly reactive, showing little variation in reaction strength, and only three and two non-reactive specimens, respectively.

Figure 23. Reactivity of recombinant TpN17 in early primary syphilis.

Western blot reaction patterns of serum specimens from 18 patients classified with early primary syphilis tested against recombinant TpN17.

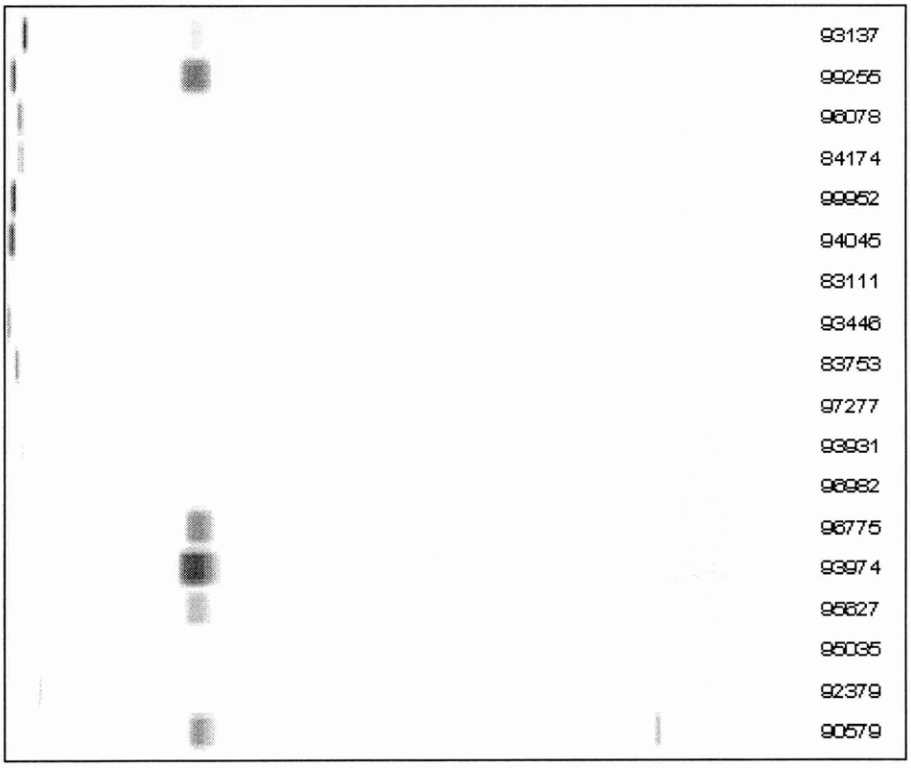


Figure 24. Reactivity of recombinant TpN17 in late primary / secondary syphilis.

Western blot reaction patterns of serum specimens from 36 patients classified with late primary syphilis or secondary syphilis tested against recombinant TpN17.

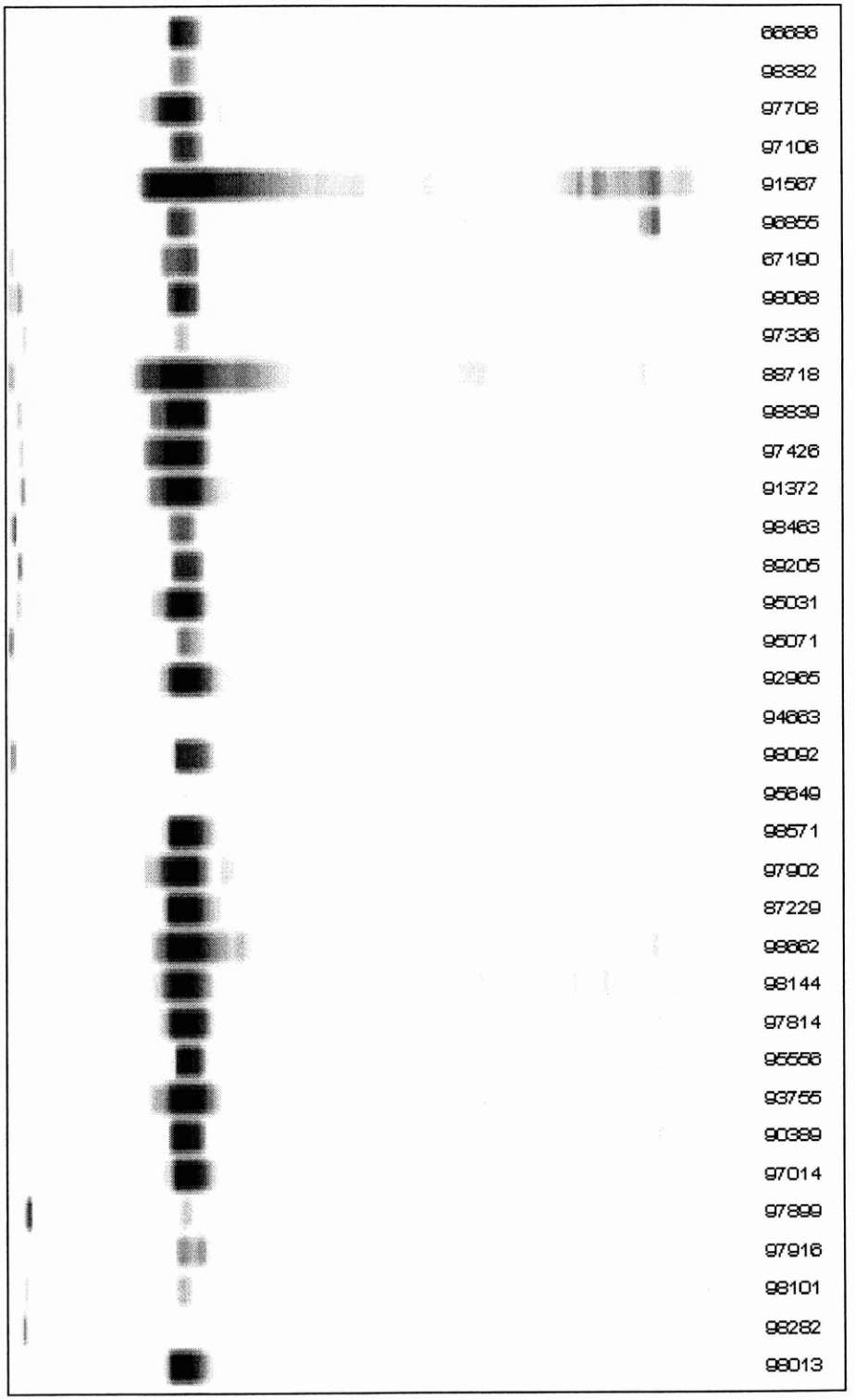


Figure 25. Reactivity of recombinant TpN17 in latent syphilis.

Western blot reaction patterns of serum specimens from 18 patients classified with latent syphilis tested against recombinant TpN17.

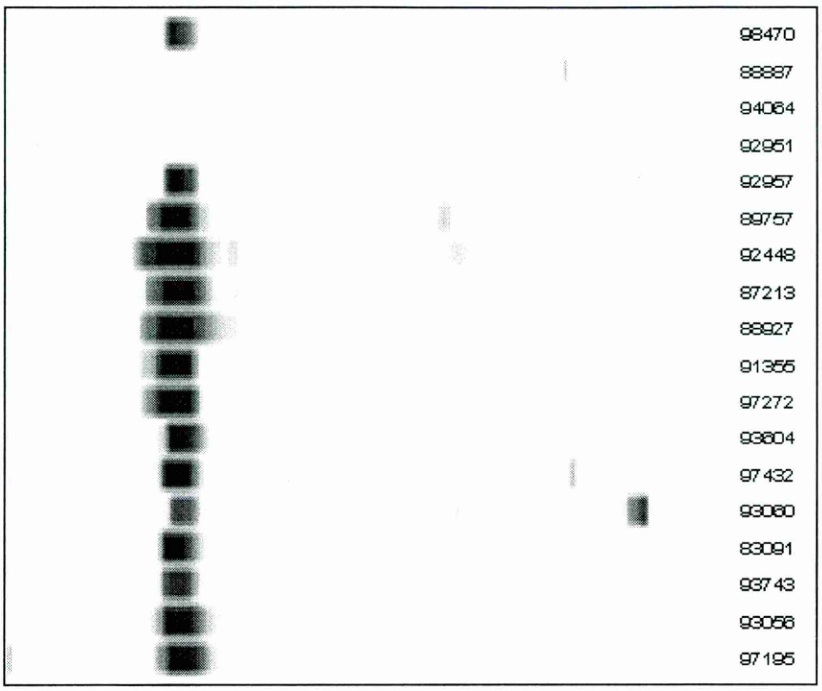
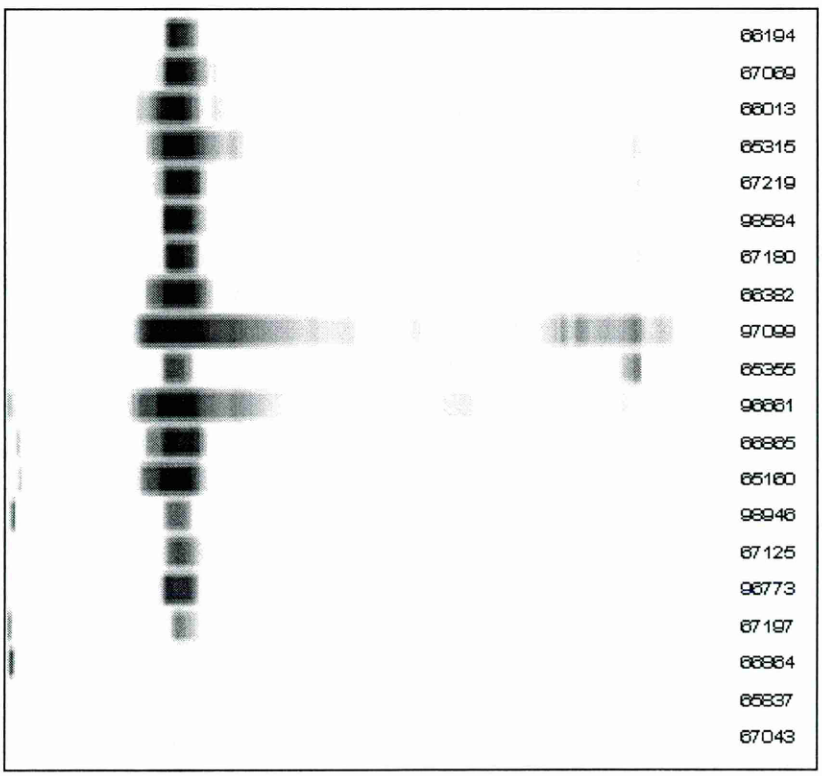


Figure 26. Reactivity of recombinant TpN17 in successfully treated cases.

Western blot reaction patterns of serum specimens from 20 patients with successfully treated infections tested against recombinant TpN17.



TpN15

The overall pattern of reactions with TpN15 are similar to those seen with TpN17, little reactivity with early primary specimens and generally strong, consistent reactions with the other categories of specimens. Fig 27 shows that most of the early primary specimens are non-reactive with only seven reactive, and then only relatively weakly. The late primary / secondary specimens (Fig 28) show the reverse situation where all, except five, are clearly reactive. Fig 29 shows that only three of the specimens from patients with latent syphilis are non-reactive and the rest show strong reactivity. This is repeated for the specimens from treated cases (Fig 30), where four are non-reactive and the rest clearly reactive.

Figure 27. Reactivity of recombinant TpN15 in early primary syphilis.

Western blot reaction patterns of serum specimens from 18 patients classified with early primary syphilis tested against recombinant TpN15.

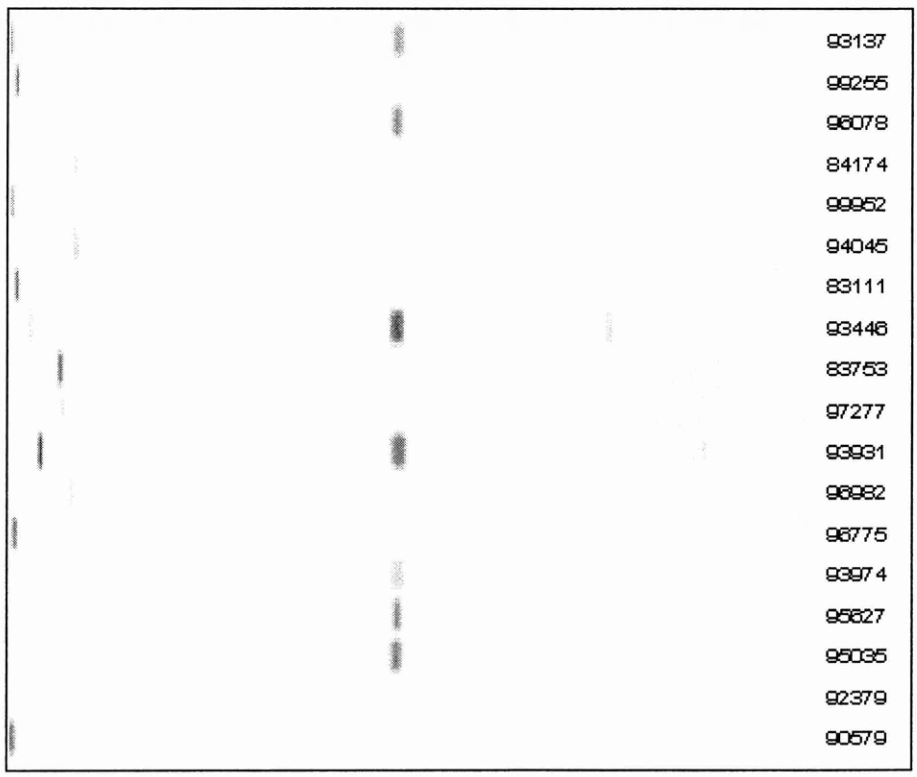


Figure 28. Reactivity of recombinant TpN15 in late primary / secondary syphilis.

Western blot reaction patterns of serum specimens from 36 patients classified with late primary syphilis or secondary syphilis tested against recombinant TpN15.

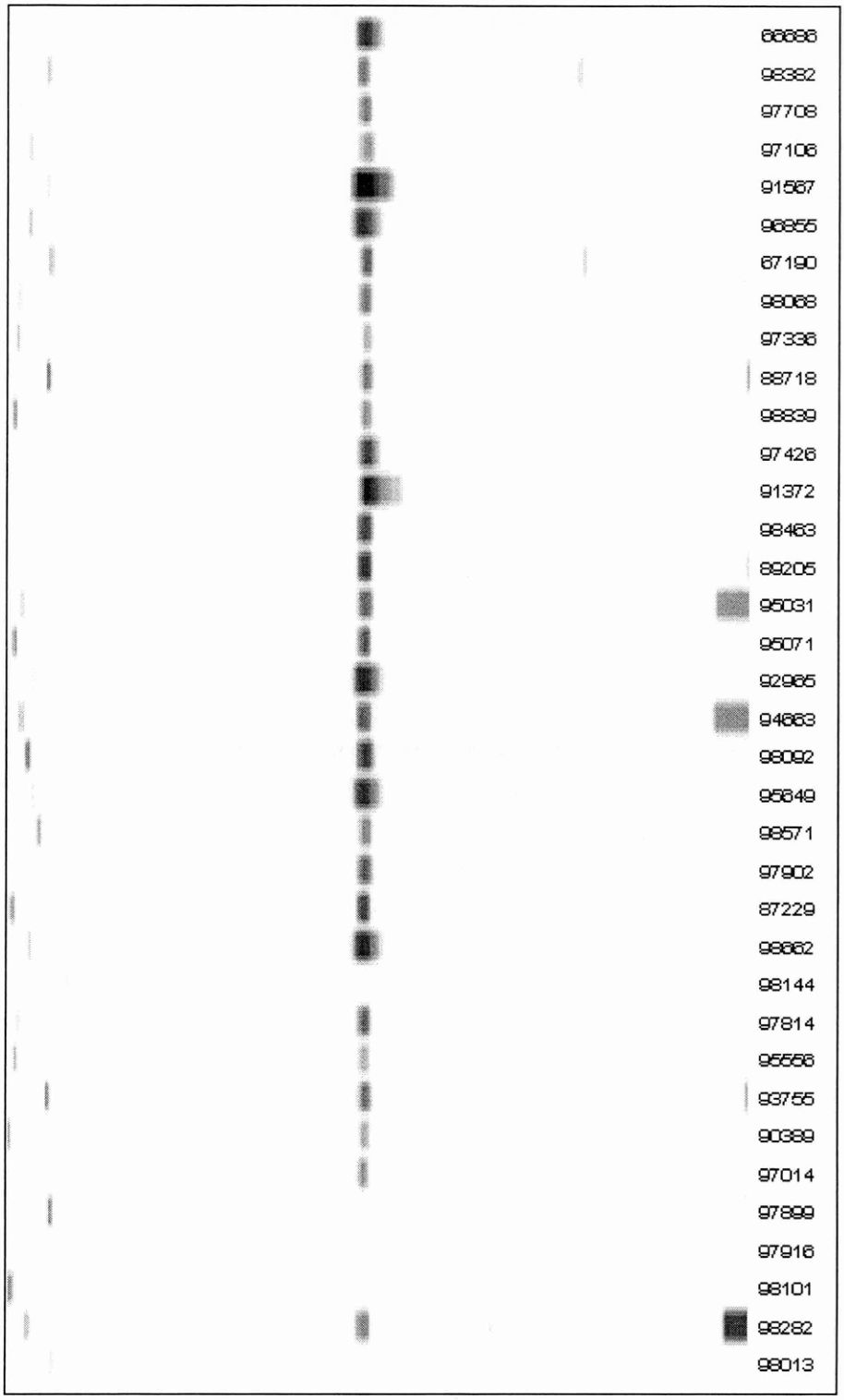


Figure 29. Reactivity of recombinant TpN15 in latent syphilis.

Western blot reaction patterns of serum specimens from 18 patients classified with latent syphilis tested against recombinant TpN15.

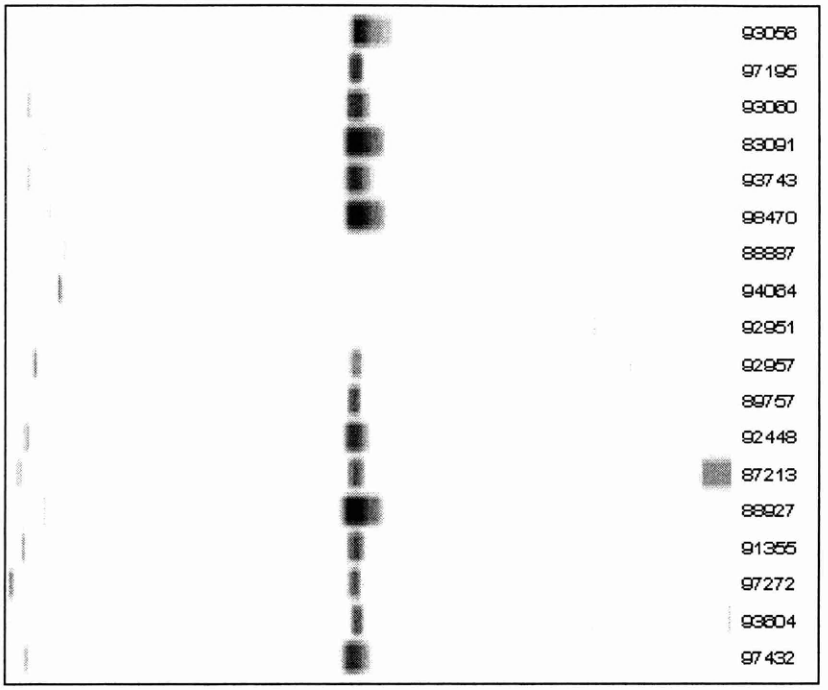
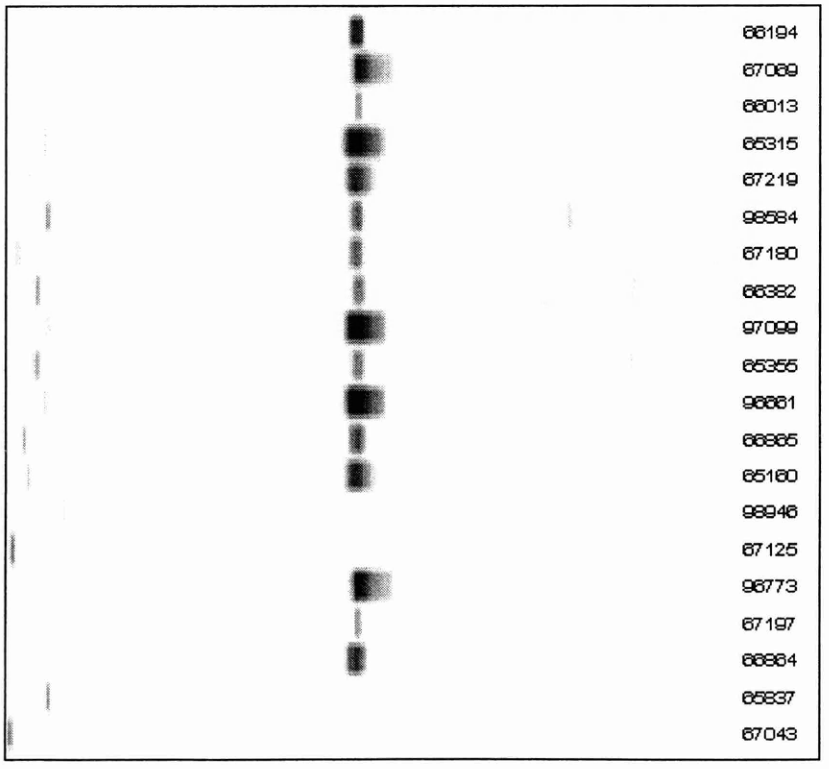


Figure 30. Reactivity of recombinant TpN15 in successfully treated cases.

Western blot reaction patterns of serum specimens from 20 patients with successfully treated infections tested against recombinant TpN15.



Discussion

The serum specimens used here were also used in the whole-organism western blots described in Chapter 3. Generally, the recombinants show an increased frequency and strength of reaction compared to the native antigen western blots. This may simply be due to the increased amount of the protein present in the blots producing greater sensitivity and to the broader bands aiding visualisation of the results. However, the underlying pattern of reactivity remains the same as that seen in the native antigen blots. The similarity in the frequency of the responses suggests that the recombinant proteins exhibit broadly similar antigenicity to that of the native antigens. Similarly, the presence of the fusion proteins does not significantly affect the frequency of the responses, and therefore probably exerts only limited effects on the antigenicity of the recombinant proteins. In early primary disease the reactivity is primarily directed toward TpN47 with significant, but weaker reaction with TpN44.5, and little reactivity to TpN15 and TpN17. In the other stages of the disease, all four antigens are frequently reactive. However, no single antigen reacted with all of the specimens tested and no reaction patterns were specific for a particular disease stage. This outcome confirms that the conclusions from the native antigen blots hold true and are not affected significantly by differences in the expression of the proteins in the native organism. It also shows TpN47 to be an important antigen for the diagnosis of syphilis, particularly in early primary infection, but although it is the antigen that most frequently elicits an antibody response, it alone is not adequate to detect all cases of syphilis. Therefore, an effective diagnostic test for detection of syphilis would probably require more than one antigen.

Summary

Generally, the recombinant proteins exhibit similar patterns of reactivity to the native antigens, and the fusion proteins do not significantly affect their antigenicity. The frequency and strength of the responses observed means that all four recombinant proteins are good candidates, individually and in combination, for the development of an enzyme immunoassay for the serodiagnosis of syphilis.

Chapter 7

Enzyme immunoassay using recombinant proteins

Introduction

The objective was to develop an enzyme immunoassay using recombinant proteins to improve the serodiagnosis of syphilis. It was essential that the method provided adequate sensitivity at all stages of the disease, especially in early primary infections. Enzyme immunoassay offers several advantages over other techniques. It is a quantitative method which is suitable for testing large numbers of specimens. An enzyme immunoassay based on antigen derived from whole organisms has proved to be highly sensitive and specific, suitable for screening, and even possibly as a confirmatory test (Young *et al.*, 1992). This assay, Captia® Syphilis-G, was shown to perform at least as well as the more widely accepted testing protocol utilising a combination of VDRL and TPHA methods. This is the main reason for using it for comparative purposes in the series of experiments described here. The results are depicted as antibody indices, which allow comparison of results between testing occasions, and are calculated by dividing the absorbance of the test specimen by the absorbance of a cut-off standard. So, values less than one represent non-reactive specimens and values greater than one are considered to be reactive, and therefore to contain antibodies to *T.pallidum*.

The use of antigen derived from natural sources imposes certain limitations on assay performance. The antigenic composition is limited to that found in the organism, so this may affect the sensitivity of the assay due to the concentrations of some antigens being sub-optimal. Alternatively, it may cause cross reactions due to the presence of antigens also expressed by other organisms. Assays based on recombinant proteins can avoid these problems, because the antigen composition and concentrations used can be optimally tailored to suit the application. The result is commonly seen as improvements in sensitivity and specificity of the recombinant assay over the native antigen based assay. Recombinant proteins have the advantage that they can be produced in relatively large quantities by *in vitro* cell culture, which is more economical than producing antigen from *in vivo* culture in animals. The quality of the antigen produced is more consistent and free from the seasonal variations seen in material from animal sources. Clearly, recombinant proteins have many advantages over native antigen preparations, which is probably why there is a steady increase in their use in all areas of serodiagnosis.

Results

TpN47

The optimal coating of the recombinant TpN47 was determined by coating curve analysis to be 1µg/mL. The resulting enzyme immunoassay showed the expected sigmoidal dose response curve with a positive specimen serially diluted with negative serum (data not shown). The coated wells were tested in triplicate with a range of serum specimens from a variety of different conditions . Fig 31 shows the absorbances of a group of serum specimens from patients with different stages of syphilis, 88 normal blood donors, and potential cross reactive samples consisting of seven patients with Lyme disease and twelve serologically confirmed biological false positives. For clarity, the normal negative specimens are shown as a "box and whisker" graphic rather than as individual values. The box extends from the 25th percentile to the 75th percentile with a horizontal line at the median, and the whiskers extend down to the smallest value and up to the largest. This shows the negatives to form a tight group with low absorbance values. The potentially cross reactive specimens all give absorbance values within the range of the negative population. The disease stage sera show a wide range of absorbances from values similar to the highest of the negative population up to the maximum response of the assay. The only significantly different distribution is the reinfection specimens, which all give high absorbances clearly separated from the negative population. Fig 32 shows a comparison of the absorbances of 76 non-selected syphilis positives obtained from a genito-urinary clinic (black) and the 88 normal blood donors (red) with their results in a commercially available enzyme immunoassay. The most obvious and notable result seen is the almost random scatter of plotted results. Some specimens that are positive in the commercial assay produce absorbance values within the range of the negative population. There are also some specimens that the commercial assay categorises as negative that produce absorbances much higher than the negative population.

Figure 31. Mean absorbances of various specimen types in an enzyme immunoassay using TpN47 coated wells.

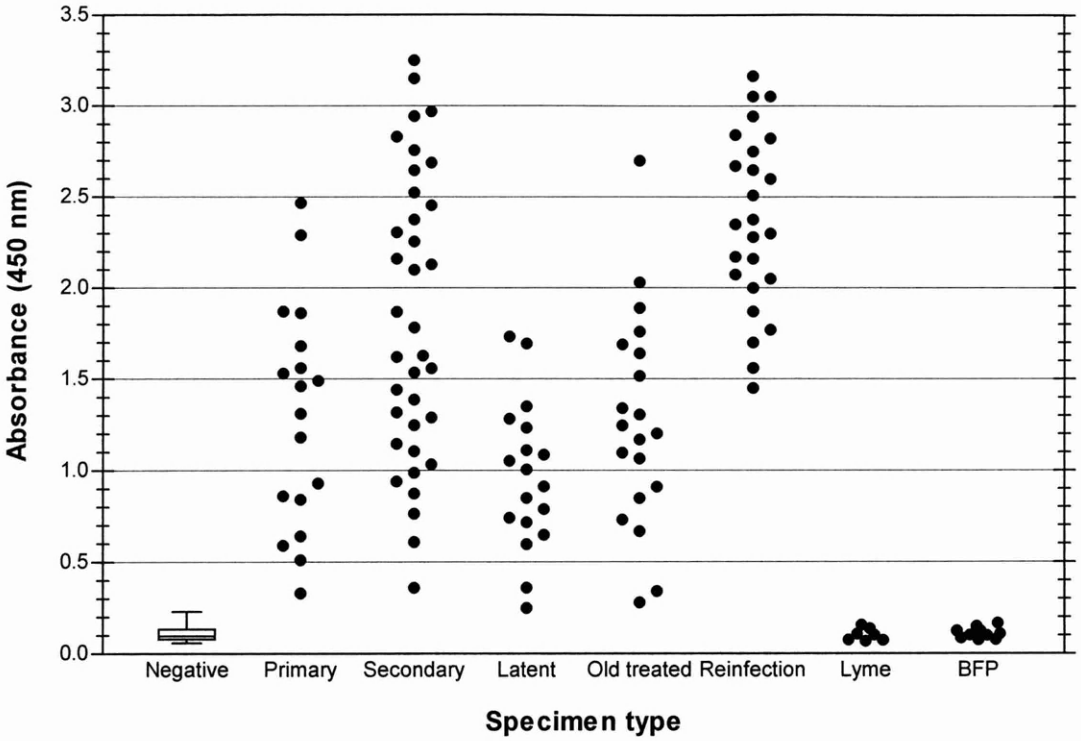
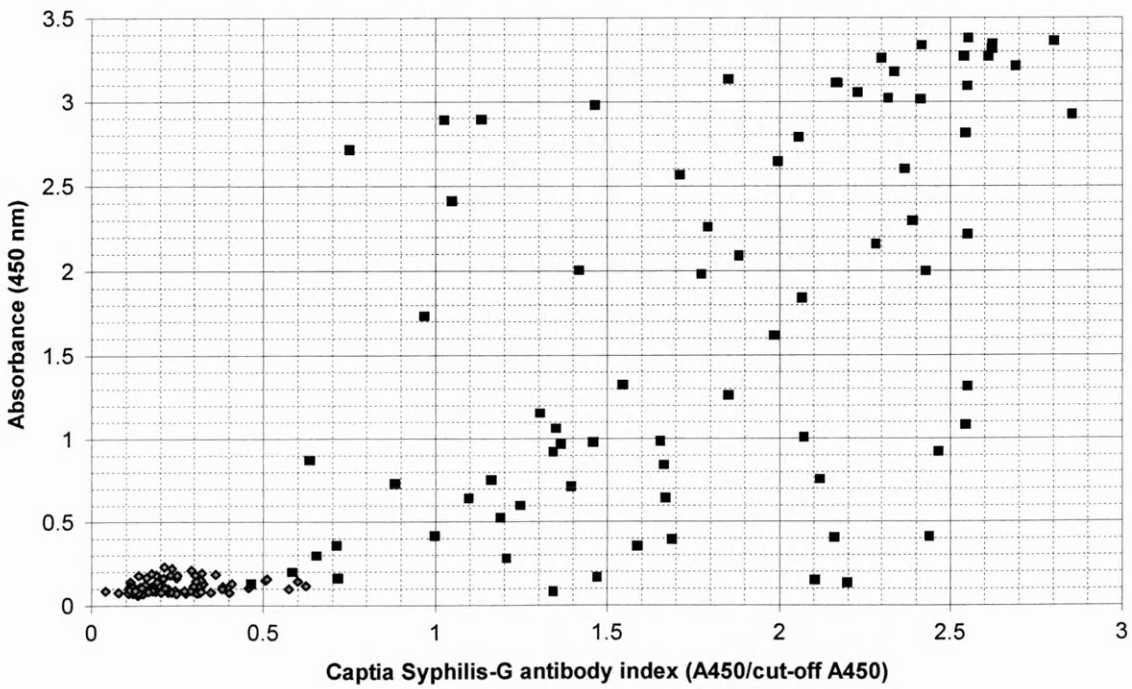


Figure 32. Comparison of an enzyme immunoassay using TpN47 coated wells with a commercially available EIA for syphilis.



TpN44.5

The optimal coating of the recombinant TpN44.5 was found to be 1µg/mL by coating curve analysis. The resulting enzyme immunoassay showed the expected sigmoidal dose response curve with a positive specimen serially diluted with negative serum (data not shown). The serum specimens described previously were tested in triplicate and the results are presented in the same format as the previous section. Fig 33 shows the generally similar results to those seen with TpN47. The negative specimens show a tight grouping with low absorbances and the cross reactive specimens give broadly similar absorbances. The syphilitic sera show a wide range of absorbance values, with a high number giving absorbances similar to those of the negative specimens. Again, the reinfection specimens produce high absorbances which are widely separated from the negative population. The result of the comparison with the commercial enzyme immunoassay is shown in Fig 34, and shows a generally similar impression to the results previously described for TpN47. The plotted results produce a wide scatter, with some specimens that are positive in the commercial assay producing absorbance values similar to the negative specimens. There are also some specimens categorised as negative by the commercial assay that produce absorbances higher than the negative population.

Figure 33. Mean absorbances of various specimen types in an enzyme immunoassay using TpN44.5 coated wells.

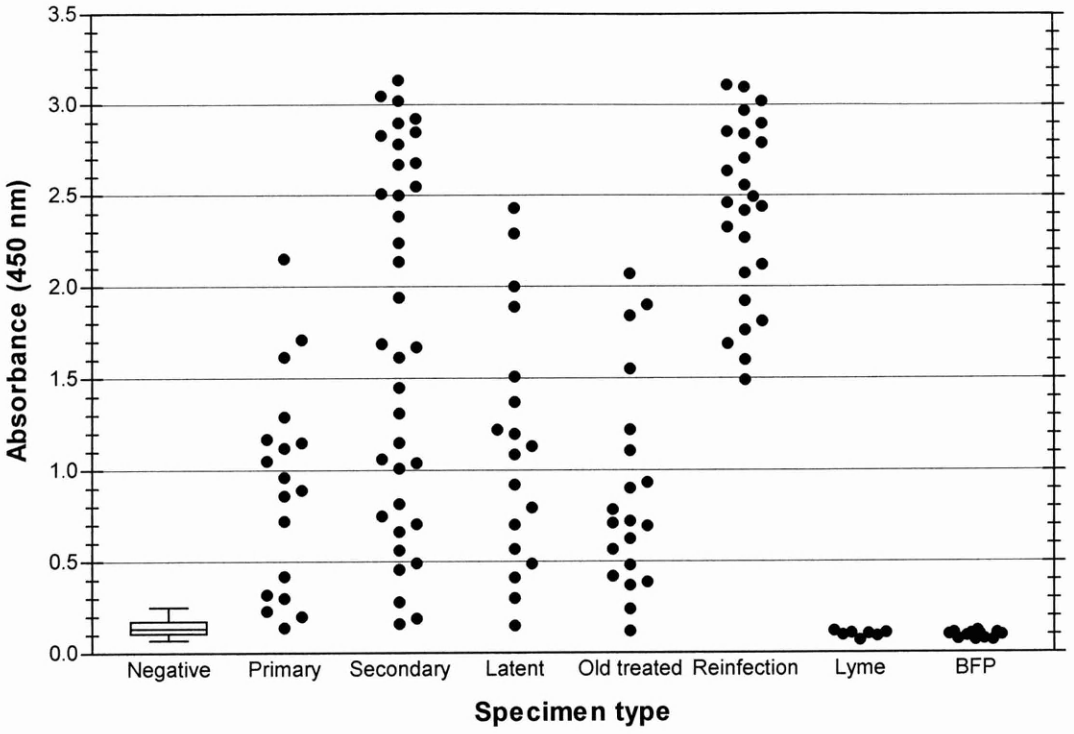
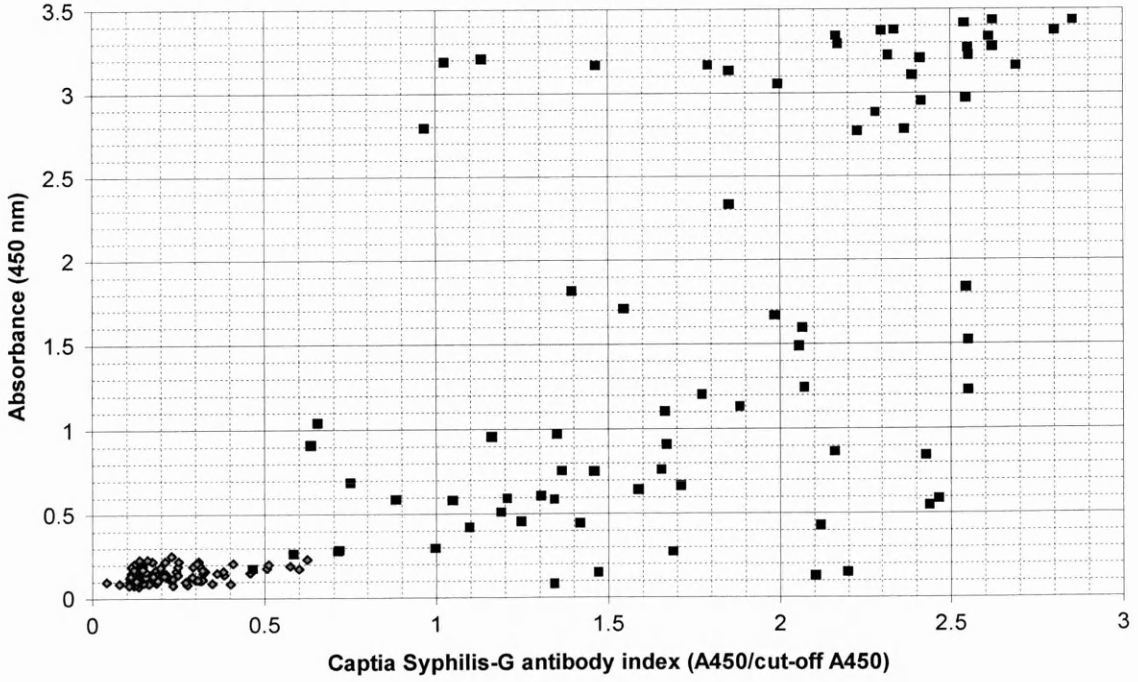


Figure 34. Comparison of an enzyme immunoassay using TpN44.5 coated wells with a commercially available EIA for syphilis.



TpN17

The optimal coating concentration for the recombinant TpN17 was determined by coating curve analysis. The wells were coated at 1µg/mL and showed the expected sigmoidal dose response curve with a positive specimen serially diluted with negative serum (data not shown). The serum specimens previously described were tested in triplicate and the results presented in the same format as the previous sections. Fig 35 shows generally similar results to those seen with TpN47 and TpN44.5, but differs in some respects. The results are similar in respect to the absorbances seen with the negative specimens and the specimens from patients with secondary, latent, treated and recurrent infections. Also, the potentially cross reactive samples again show absorbances within the negative range. The difference is seen in the results of the specimens from patients with early primary disease which show generally much lower absorbances than were seen when they were tested with TpN47 and TpN44.5. Fig 36 depicts the result of the comparison with the commercial enzyme immunoassay, and shows a generally similar pattern to the results previously described. The plotted results produce a wide scatter, with some specimens that are positive in the commercial assay producing absorbance values similar to the negative specimens. There are also some specimens categorised as negative by the commercial assay that produce absorbances higher than the negative population.

Figure 35. Mean absorbances of various specimen types in an enzyme immunoassay using TpN17 coated wells.

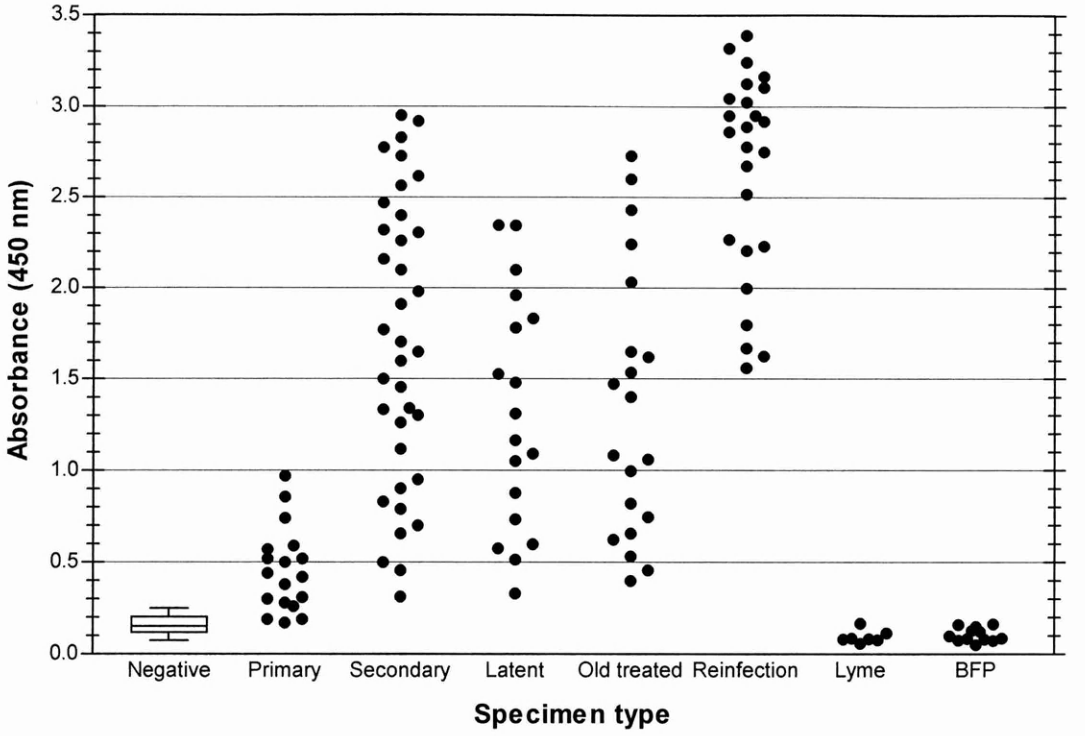
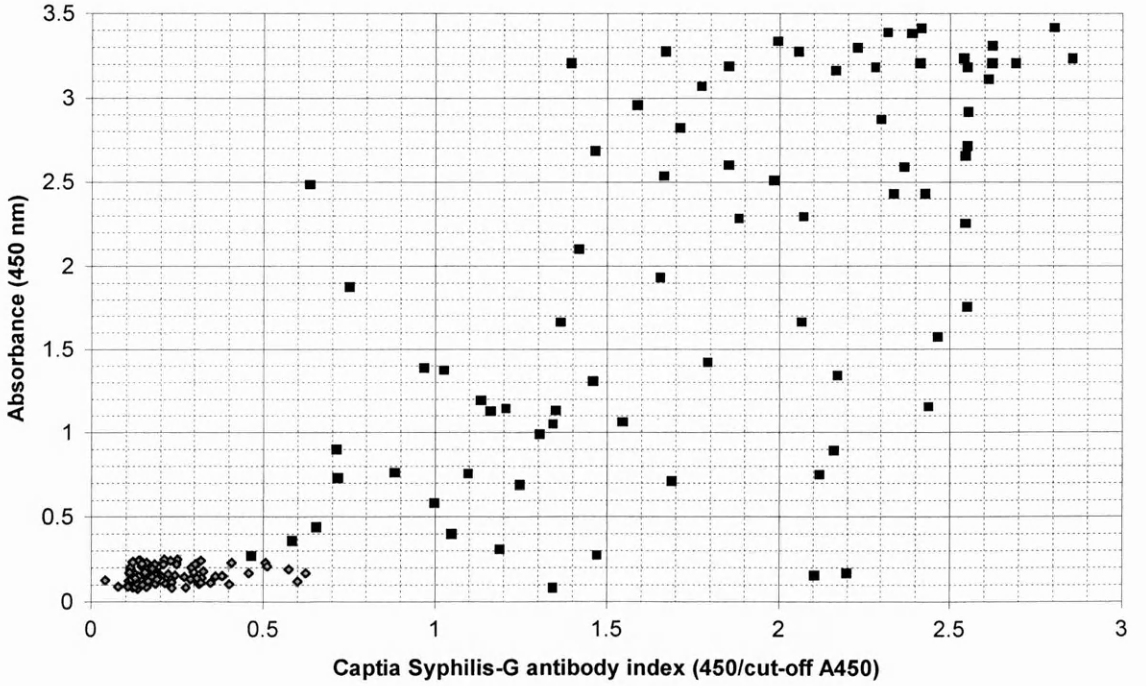


Figure 36. Comparison of an enzyme immunoassay using TpN17 coated wells with a commercially available EIA for syphilis.



TpN15

Coating curve analysis was used to determine the optimal coating concentration for the recombinant TpN15. The coated wells were coated at 1µg/mL and showed the expected sigmoidal dose response curve with a positive specimen serially diluted with negative serum (data not shown). The serum specimens previously described were tested in triplicate and the results presented in the same format as the previous sections. Fig 37 shows the results to be generally similar to those seen with TpN17. The negative specimens produce low absorbances in a narrow range. The early primary specimens give generally lower absorbances than the other syphilitic sera and several produce results within the range of negative values. The other syphilitic sera produce a range of absorbances covering the full range of the assay, except the reinfection samples which showed predominantly high absorbances. Again, the specimens from patients with Lyme disease and the biological false positive samples produced results within the range of the negative specimens. Fig 38 depicts the result of the comparison with the commercial enzyme immunoassay, and shows a slightly different pattern to the distribution of results to those previously described. The plotted results still produce a scatter, but it tends to be mostly directed towards specimens that are positive in the commercial assay producing absorbance values similar to the negative specimens. However, there are two specimens categorised as negative by the commercial assay that produce absorbances higher than the negative population.

Figure 37. Mean absorbances of various specimen types in an enzyme immunoassay using TpN15 coated wells.

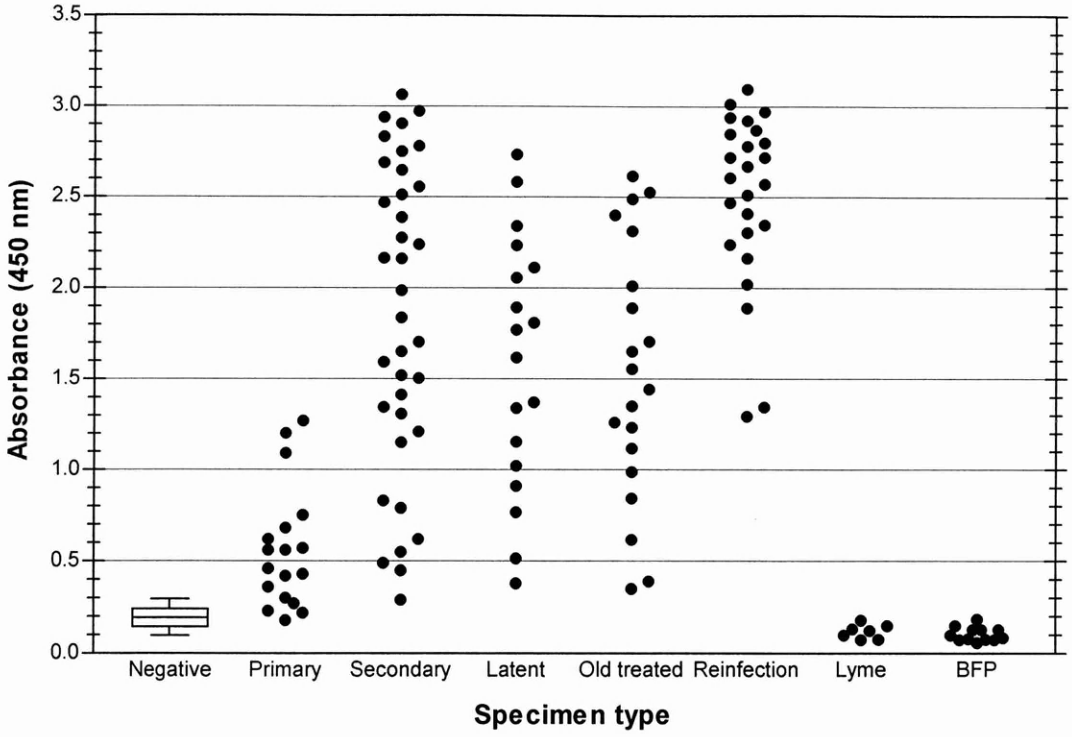
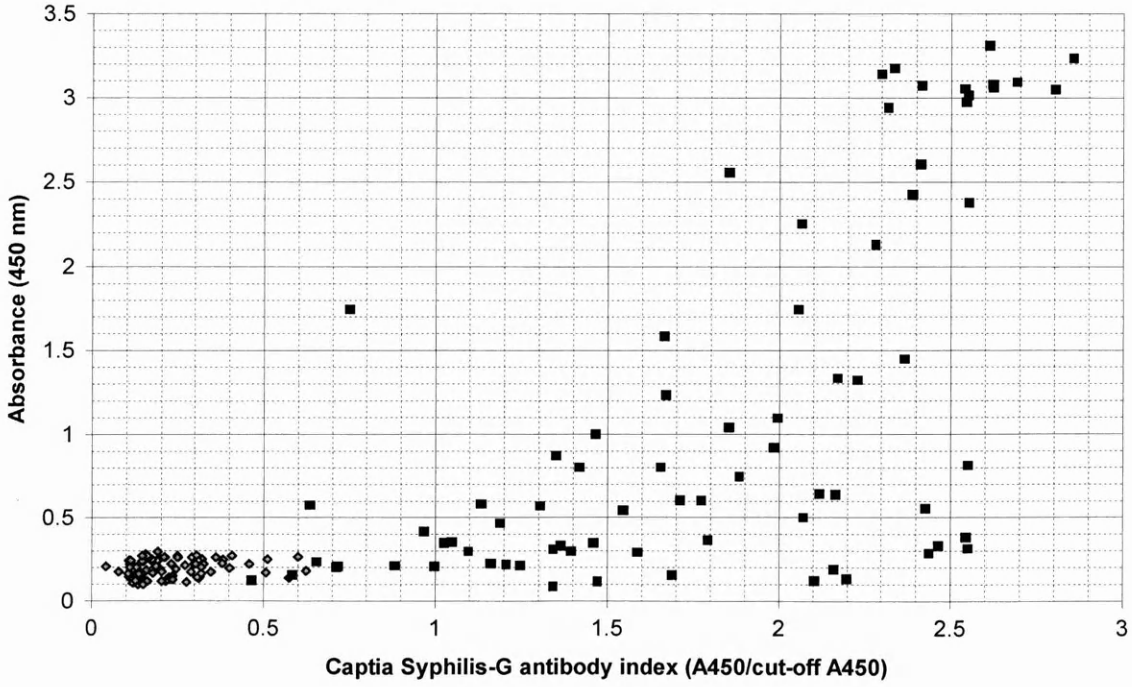


Figure 38. Comparison of an enzyme immunoassay using TpN15 coated wells with a commercially available EIA for syphilis.



Combined coating

The coating concentrations within the mixture of each of the recombinant proteins was determined using ECHIP experimental design software to formulate an experiment using a Mixture design. Response surface analysis showed there to be distinct interactions between the components of the mixture, but a broad plateau in the response covered the central region of the experimental space. The four recombinants were coated at equal concentrations with a total coating concentration of 1µg/mL, i.e. four recombinants each at 0.25µg/mL. The resulting enzyme immunoassay showed the expected sigmoidal dose response curve with a positive specimen serially diluted with negative serum (data not shown). The coated wells were tested in triplicate with a range of serum specimens and the results are presented in the same format as the previous sections. Fig 39 shows that the negative specimens and the potential cross reactive samples produce lower, more tightly grouped absorbances than was seen with any of the recombinant proteins coated singly. The syphilitic specimens show a range of absorbances, but now the lowest absorbances are significantly higher than the most reactive negative specimens. Fig 40 depicts the result of the comparison with the commercial enzyme immunoassay, and shows a slightly different pattern to the distribution of results to those previously described. The plotted results produce a much tighter grouping through the center diagonal of the graph. There are some specimens categorised as negative by the commercial enzyme immunoassay that produce results significantly greater than the negative population. There are no specimens giving antibody indices greater than one in the commercial assay that produce absorbances even close to the negative range.

Figure 39. Mean absorbances of various specimen types in an enzyme immunoassay using wells coated with an optimal combination of all four recombinants.

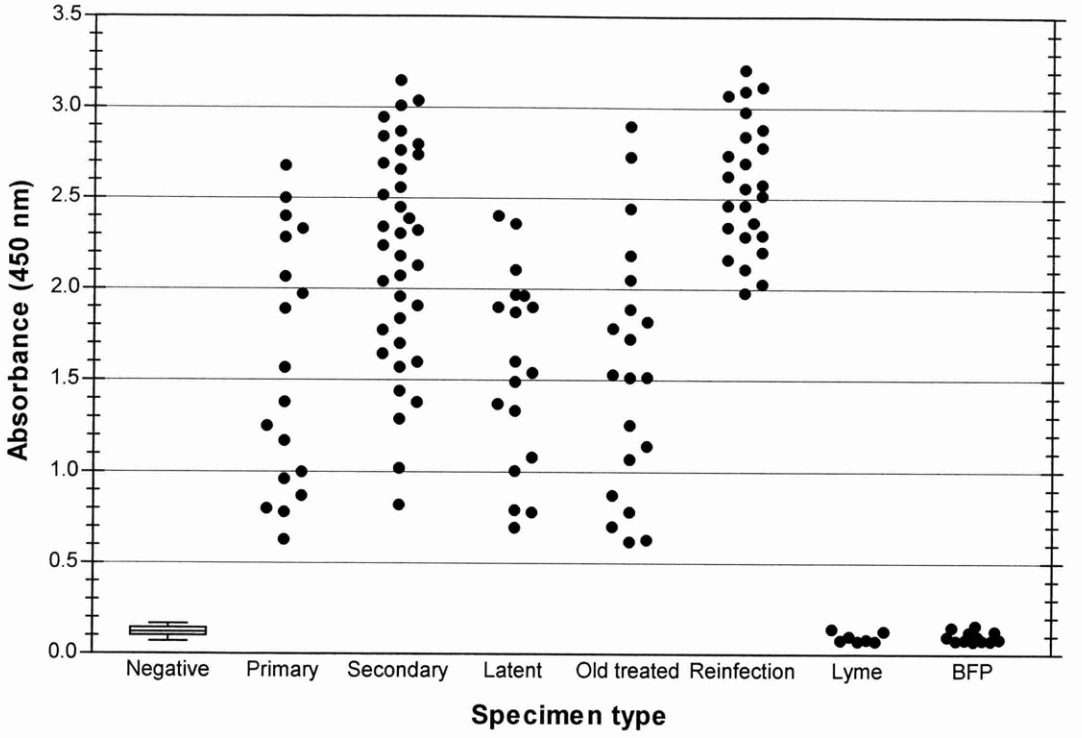
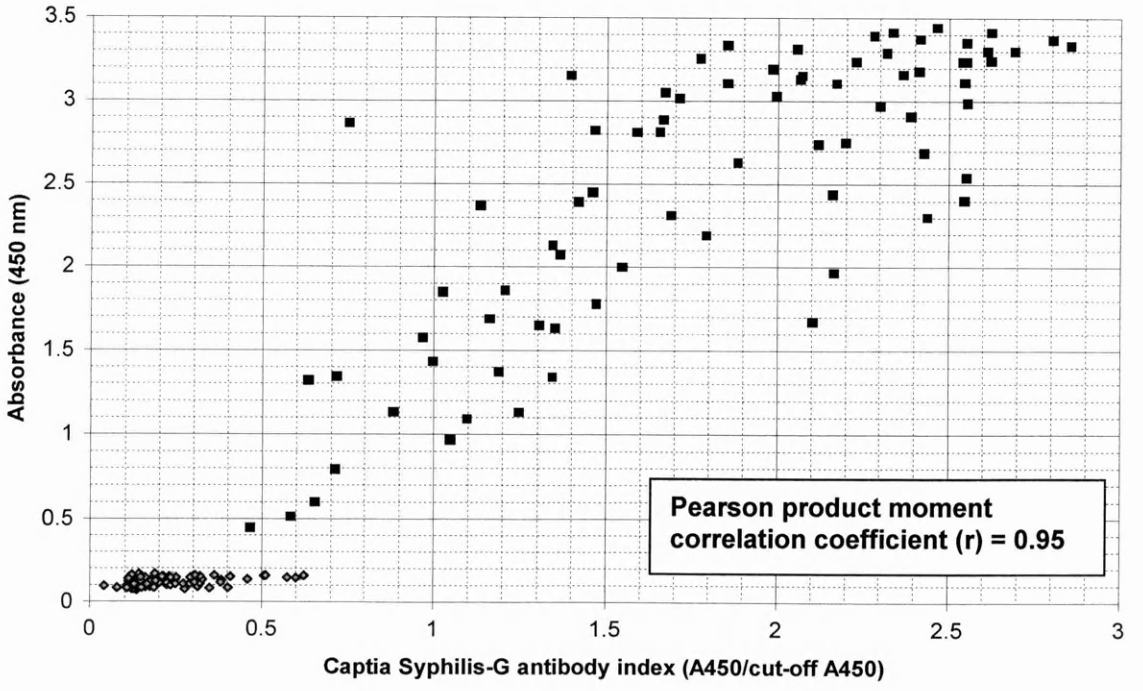


Figure 40. Comparison of an enzyme immunoassay, using wells coated with an optimal combination of all four recombinants, with a commercially available EIA for syphilis.



Discussion

Generally, the assays produced by the recombinant proteins coated singly each give a compact negative distribution which suggests good specificity. The lack of reactivity with the samples from patients with Lyme disease and the biological false positive specimens also indicates the specific nature of the responses. They produce good responses with most of the positive specimens, but all to a greater or lesser extent fail to adequately discriminate some of the lower responding specimens from the negative population. This picture is characteristic of assays that lack sensitivity, thereby falsely categorising a proportion of the specimens as negative. The combined coating produces an assay with an even more compact distribution to the negative specimens than any of the single antigen coatings achieved. There is also an increase in the reactivity of the lowest reacting positive specimens relative to the negative population. These findings suggest the combined coating produces an assay that is more sensitive than any of the individual antigen assays, and is probably more specific as well. These two effects combine to produce greater separation between the lowest reacting specimens and the most reactive negative specimens. This means that the combined coating produces an assay with the necessary discrimination to differentiate reliably between positive and negative specimens. This outcome shows the limitations associated with using single antigens and clearly demonstrates the benefits of using a combined coating for the detection of antibodies to *T.pallidum*.

The evaluation above indicates the superiority of using a combination of four antigens, but it does not give any information on how these results relate to the current serological testing practices, i.e. quantitative data on the performance relative to other immunoassays. The results were compared with those of a leading commercially available immunoassay, Captia® Syphilis-G, based on wells coated with antigen derived from whole *T.pallidum*. It has been extensively evaluated and shown to be extremely sensitive and specific (Lefevre *et al.*, 1990; Young *et al.*, 1989; Young *et al.*, 1992; Ross *et al.*, 1991; Backhouse and Hudson, 1995; Hooper *et al.*, 1994; Nayar and Compas, 1993). It has been recommended for screening and is used by many blood centres for routine testing. It has also been suggested that it may be suitable for confirmatory testing in place of FTA-ABS for positive results identified by other methods. These many evaluations mean that the performance characteristics of the assay are well known and documented, so it provides a good assay for comparative purposes. The results with the specimens from the genito-urinary clinic, when tested in

the single antigen enzyme immunoassays, basically reiterated the findings of the disease stage-characterised specimens. The absorbances of the positive specimens were widely spread with many overlapping the negative population. Additionally, they show poor correlation with the Captia® Syphilis-G antibody index producing a scatter of results when plotted. The overlap of the positive specimens with the negative population is probably simply due to poor sensitivity of the assay, but the scattering of the results seen suggests an additional phenomenon is present. A simple lack of sensitivity would be manifested as a scatter of results around a line of equivalence with good correlation, but with a low slope. Specimens with strong responses in the Captia® Syphilis-G assay would produce much lower absorbances in the recombinant antigen assay, but all the specimens would maintain their relationships to each other. In the scattering of the results observed here some strong Captia® Syphilis-G positives are poorly reactive in the recombinant immunoassay, yet others of a similar reactivity are strongly reactive. This suggests that each of the individual recombinant proteins fails to detect significant antibody responses in some specimens that are detected by the whole treponeme based assay. The antibody responses of the specimens to each of the recombinant proteins are not directly proportional to their total anti-*T.pallidum* activity. This means that any assay based on a single recombinant protein would tend to show poor sensitivity and give more false negative results than the commercial immunoassay. Some of the scattering of the results seen here is caused by a proportion of the specimens being detected more effectively by the recombinant proteins than by native antigen. This is probably due to the increase in the amount of antigen present when recombinant proteins are used to coat the solid phase, thus increasing sensitivity to that particular antigen.

When the specimens were tested against a combined coating of the four recombinants, the data generally replicated the findings previously seen. The range of absorbances of the negative specimens was reduced, producing a much tighter distribution, and the absorbances of the positive specimens were generally increased. Some of the specimens just below the Captia® Syphilis-G cut-off were now shown to be distinctly more reactive than the negative population. These specimens probably contain low levels of syphilis antibodies and were falsely categorised as non reactive, suggesting that the combined coating produces an assay with greater sensitivity than the commercial immunoassay. Another noticeable effect was the improved correlation between the recombinant immunoassay absorbance and the Captia® Syphilis-G antibody index, shown by a

reduction in the spread of the positive results. This suggests that the antibodies detected by the combined coating are a proportionate sample of those detected by the whole cell assay and no significant reactivities are left undetected. The combined coating of the four recombinant proteins performs well and produces an assay with a tight negative distribution and a good dose response. The assay discriminated well between positive and negative specimens, and is potentially more sensitive than the commercial immunoassay used for comparison.

Studies have shown antigenic cross-reactivity between *T.pallidum* and the non pathogenic Reiter's spirochaete, *T.phagedenis* (Pedersen *et al.*, 1980. Pedersen *et al.*, 1981. Lukehart *et al.*, 1982. Wos and Wicher, 1986. Baker-Zander and Lukehart, 1984). This cross reactivity has been widely exploited in syphilis serology in the use of *T.phagedenis* as the antigen source in the Reiter protein complement fixation test. It has also been used as a sorbent in the FTA-ABS test to remove cross reacting antibodies. *T.phagedenis* has been shown to encode and express a homologue of TpN44.5 (Yelton *et al.*, 1991). This suggests the possibility that the use of TpN44.5 as an antigen might produce unwanted cross reactions. However, an enzyme immunoassay based on TpN44.5 has been shown to be highly specific and sensitive, and comparable in performance to TPHA (Ijsselmuiden *et al.*, 1989a). The only limitation of the test was a slight lack of sensitivity in early primary disease, which is a ubiquitous problem in syphilis serology. There was no evidence of cross reactivity in this study, so cross reactions may be more of a theoretical problem than a reality. Reviewing the data obtained for individual sera in this study shows that no specimen is reactive solely with TpN44.5, so omitting it from the combined coating may not be detrimental to the assay performance. This suggestion is supported by a recent publication (Young *et al.*, 1998) evaluating a new commercial immunoassay based on a combined coating of recombinant TpN47, TpN17 and TpN15. This study shows the assay to have superior sensitivity to Captia® Syphilis-G, particularly with early primary infections and patients with HIV co-infection. Perhaps elimination of TpN44.5 from the antigen coating may not have any detrimental effects on overall assay sensitivity.

In conclusion, a combined coating of the four recombinant proteins has been shown to be suitable for the serological diagnosis of syphilis, but a coating of TpN47, TpN17 and TpN15 may be equally effective and should be investigated. The role of TpN44.5 should be investigated further to determine if its inclusion is detrimental or bestows any benefits on the assay performance.

Summary

The enzyme immunoassays using each of the recombinant antigens individually lack sensitivity and the scattering of the results suggests that they each miss key antibody responses that are detected by the assay based on antigen derived from whole organisms. The combination of all four recombinant antigens produces an assay with better sensitivity than the native antigen assay. This is shown by the significant separation of the most reactive negative specimens from the least reactive syphilitic specimen. Additionally, there is much better correlation between the absorbances of the syphilitic specimens and their antibody indices in the native antigen assay. This suggests that all the significant antibody specificities are detected by the combination of the four recombinant proteins. The enzyme immunoassay developed here has the potential to perform well in a clinical situation, but requires additional testing to confirm its specificity and allow a cut-off to be determined.

Chapter 8

Synthetic peptide epitope mapping

Introduction

Although recombinant proteins have many advantages over antigen derived from animal sources, they are still quite time consuming to produce and purify. There is also the problem of residual *E.coli* proteins, which are a potential cause of false positive results. This is exaggerated when several recombinant proteins are required to provide the necessary antigenicity. If the antigenic sequences within the proteins were identified, then perhaps a single synthetic recombinant protein could be constructed that consisted of a concatenation of these sequences. This would considerably reduce the complexity of the assay design. Depending on the number and strength of the antigenic sequences, an alternative strategy might be to produce synthetic peptides that represent the antigenic sequences of the proteins. Synthetic peptides can be produced to high levels of purity on an industrial scale, and are relatively inexpensive diagnostic reagents due to the low coating concentrations required.

The initial objective was to identify the antigenic sequences that the antibody responses were directed towards, using a synthetic peptide epitope mapping technique. Synthetic peptide epitope mapping is a simple way to define the linear or sequential epitopes within a protein sequence. However, due to restricted lengths of the peptides the technique fails to identify non-sequential or conformational types of epitope. The technique uses a set of overlapping peptides of a defined length, each offset from the previous peptide by a chosen number of residues, which are homologous to the amino acid sequence of the native protein. The peptide length and offset chosen are usually a compromise between the cost of the synthesis and the resolution of the experiment required. The use of short peptides with large overlaps and small offsets provides the highest resolution, but can be prohibitively expensive. For longer protein sequences, where cost of synthesis is a major consideration, a larger offset of 2 or 4 residues can be used. This reduces the number of peptides required, but also reduces the resolution of the experiment. However, this can be compensated for, to some extent, by using longer peptides, which reduces the chances of bisecting epitopes. TpN47 and TpN44.5 were mapped using a set of 14-mer peptides each offset from the previous one by four residues. TpN17 was mapped with 12-mer peptides offset by three residues, and TpN15 with 10-mer peptides offset by two residues. This technique is based on the simultaneous synthesis of large numbers of peptides using standard Fmoc chemistry. Synthesis of the peptides on derivatised pins was accomplished by repetitive cycles of Fmoc-deprotection,

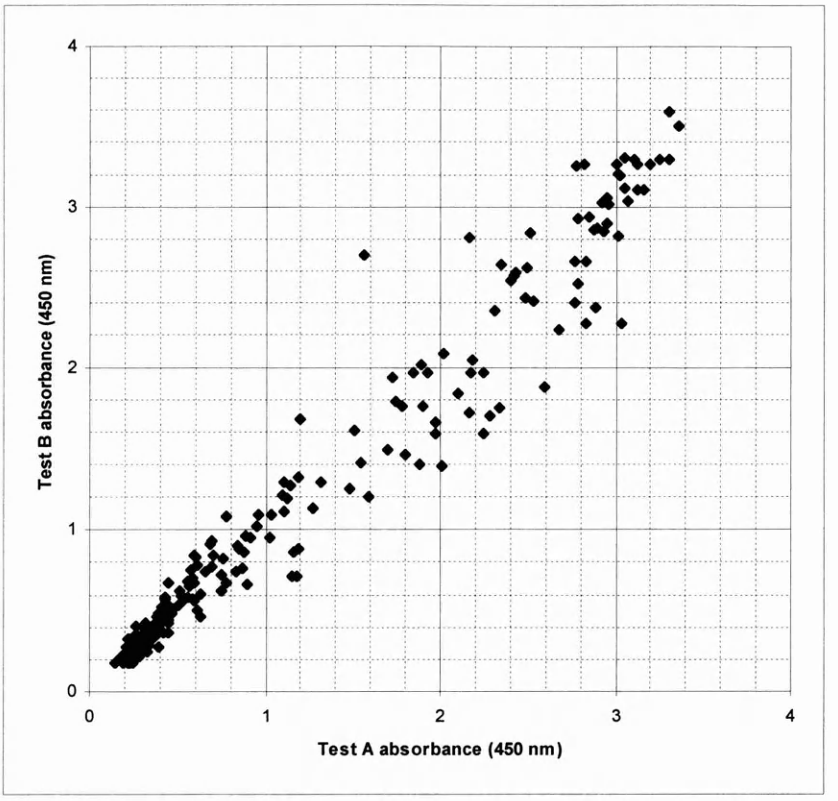
washing and amino acid coupling, adding one amino acid residue per cycle. After completion of synthesis of the desired peptides, the final Fmoc-protecting group was removed and the terminal amino group capped by acetylation. The side-chain protecting groups were removed by trifluoroacetic acid treatment. The success of the experiment depends on the correct synthesis of the peptides, and to ensure this various controls were incorporated during the synthesis procedure. Bromophenol blue stains the free amino groups blue, and was added to the coupling mixture to monitor the addition of each amino acid to the peptide. Successful coupling through the deprotected amino groups causes bleaching of the colour due to their reaction and elimination. Therefore, after each round of coupling the pins should become colourless, confirming successful attachment of the next amino acid in the chain. The peptide PLAQ reacts specifically with a control monoclonal antibody supplied with the derivatised pins, and the tetrapeptide GLAQ fails to react. These two control peptides were synthesised on each block of pins, in parallel with the test peptides. After synthesis, the reactions of the control peptides with the monoclonal antibody were compared to the reactions of identical peptides synthesised and supplied by the manufacturer. Comparable reactivity to the manufacturer-supplied peptides indicates successful synthesis has been achieved. The overlapping peptide sets were tested with specific antisera using an enzyme immunoassay procedure to identify antigenic regions and locate epitopes within the protein.

Results

The absorbances of the control peptides, synthesised in parallel to the test peptides, closely matched those of control peptides supplied by the manufacturer. All the test peptides produced low absorbances, less than 0.1, when tested directly with the monoclonal anti-human IgG/HRP conjugate. Repetition of this test after every five specimens tested produced similar low absorbances with all peptides. The testing of the peptides was further controlled by periodically testing the same syphilitic serum specimen. Fig 41 shows a graph of the absorbances from the first (test A) and last (test B) of these tests plotted against each other. The absorbances correlate well in terms of relative reactivities and absolute absorbance values, resulting in a narrow scatter of points around the x= y line. This is confirmed by regression analysis of the data which produces an R^2 value of 0.98 and a slope of 0.99.

Figure 41. Effect of multiple cycles of testing and regeneration on peptide activity.

Absorbance of the peptides tested with a syphilitic serum at the beginning of the study (test A) compared with the same specimen retested at the completion of the testing programme (test B).



The peptides were tested with serum specimens from 18 normal blood donors, 22 patients with syphilis, and a pool of syphilitic serum before and after affinity purification. A cut-off value (mean + 3 SD of the 100 least reactive peptides) was applied to the data to identify the most significant reactivities. Peptides above the cut-off are identified in red and those below in blue. All serum specimens from normal blood donors generally gave absorbances of less than 0.1 with all peptides. A typical set of results are shown in Figure 42. When the peptides were tested with serum specimens from patients with syphilis some peptides showed more reactivity than was typically seen with a negative specimen and there was a range of absorbances across the peptides producing reactive regions interspersed with less reactive sequences. The overall reactivity of each serum specimen varied greatly. Fig 43 shows the epitope scans from a weakly reacting specimen where the range of responses across the peptides is relatively small. Fig 44 shows the other end of the spectrum of the responses seen where there is a large difference in absorbance between the most reactive and least reactive peptides. This type of response produces clearly defined reactive regions separated by several non reactive peptides.

Figure 42. Epitope scans produced by a serum specimen from a normal blood donor.

A; Absorbances of the set of 14-mer overlapping peptides covering TpN47. B; Absorbances of the set of 14-mer overlapping peptides covering TpN44.5. C; Absorbances of the set of 12-mer overlapping peptides covering TpN17. D; Absorbances of the set of 10-mer peptides covering TpN15.

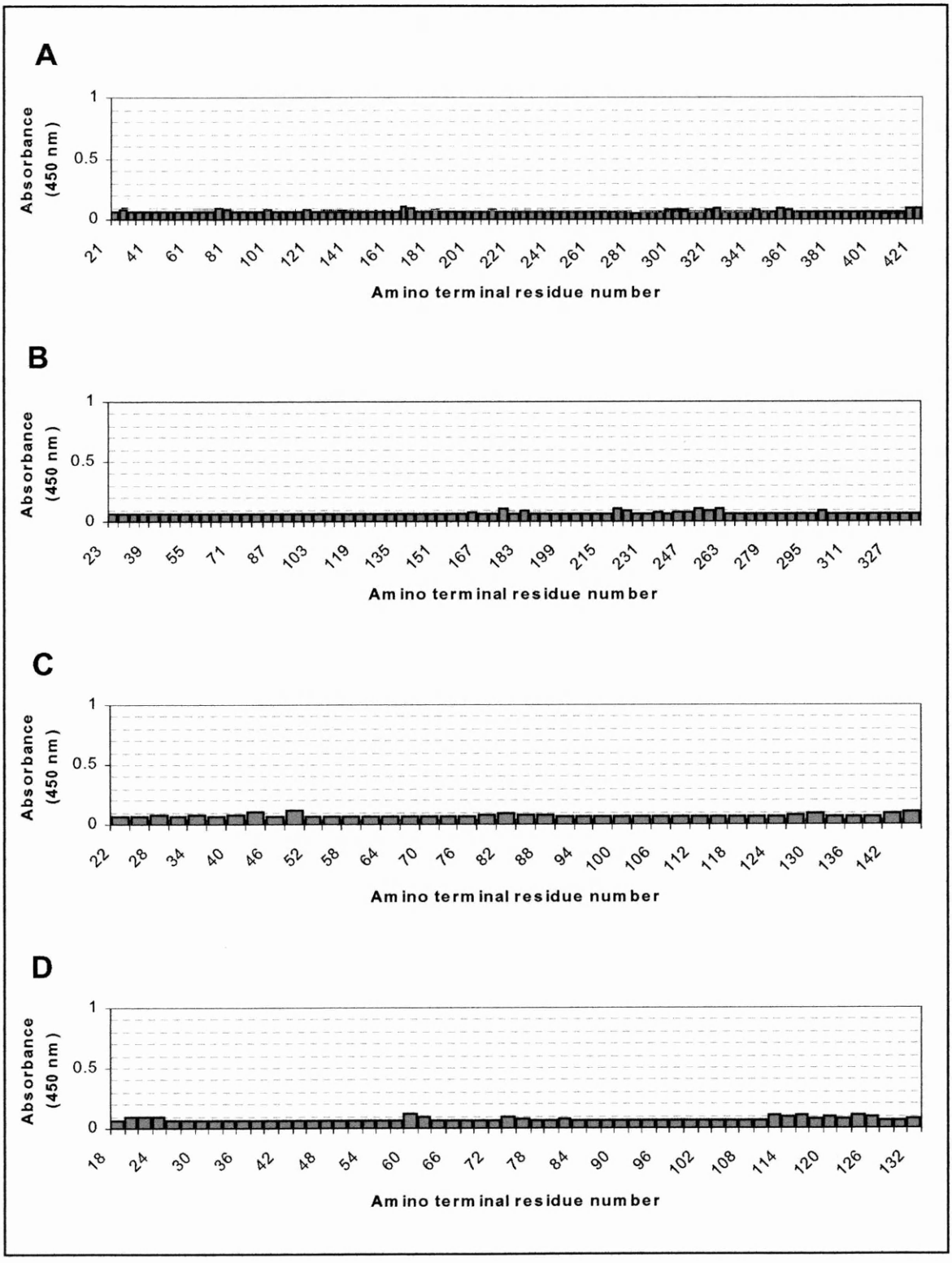


Figure 43. Epitope scans produced by a weakly reacting serum specimen from a patient with syphilis.

A; Absorbances of the set of 14-mer overlapping peptides covering TpN47. B; Absorbances of the set of 14-mer overlapping peptides covering TpN44.5. C; Absorbances of the set of 12-mer overlapping peptides covering TpN17. D; Absorbances of the set of 10-mer peptides covering TpN15.

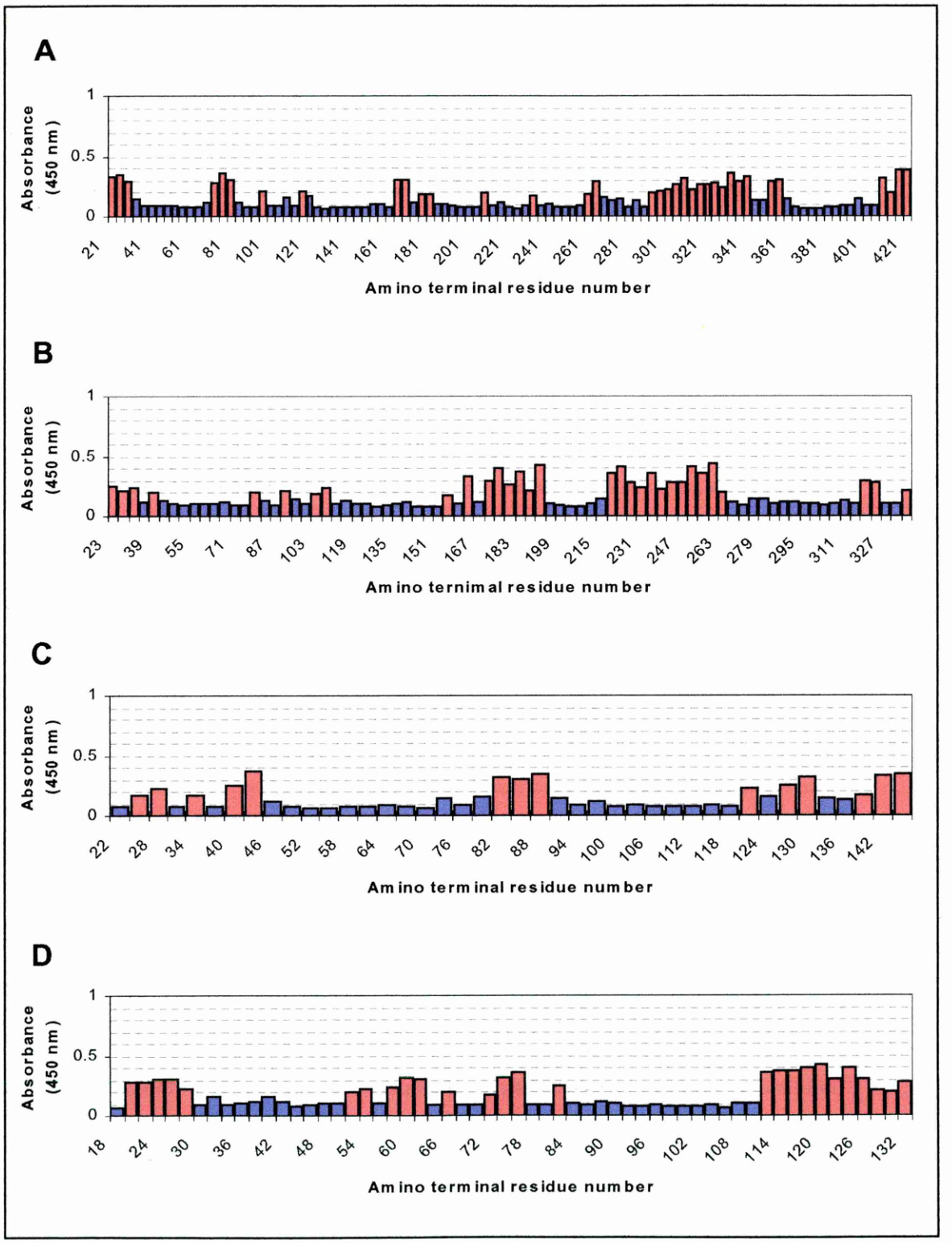
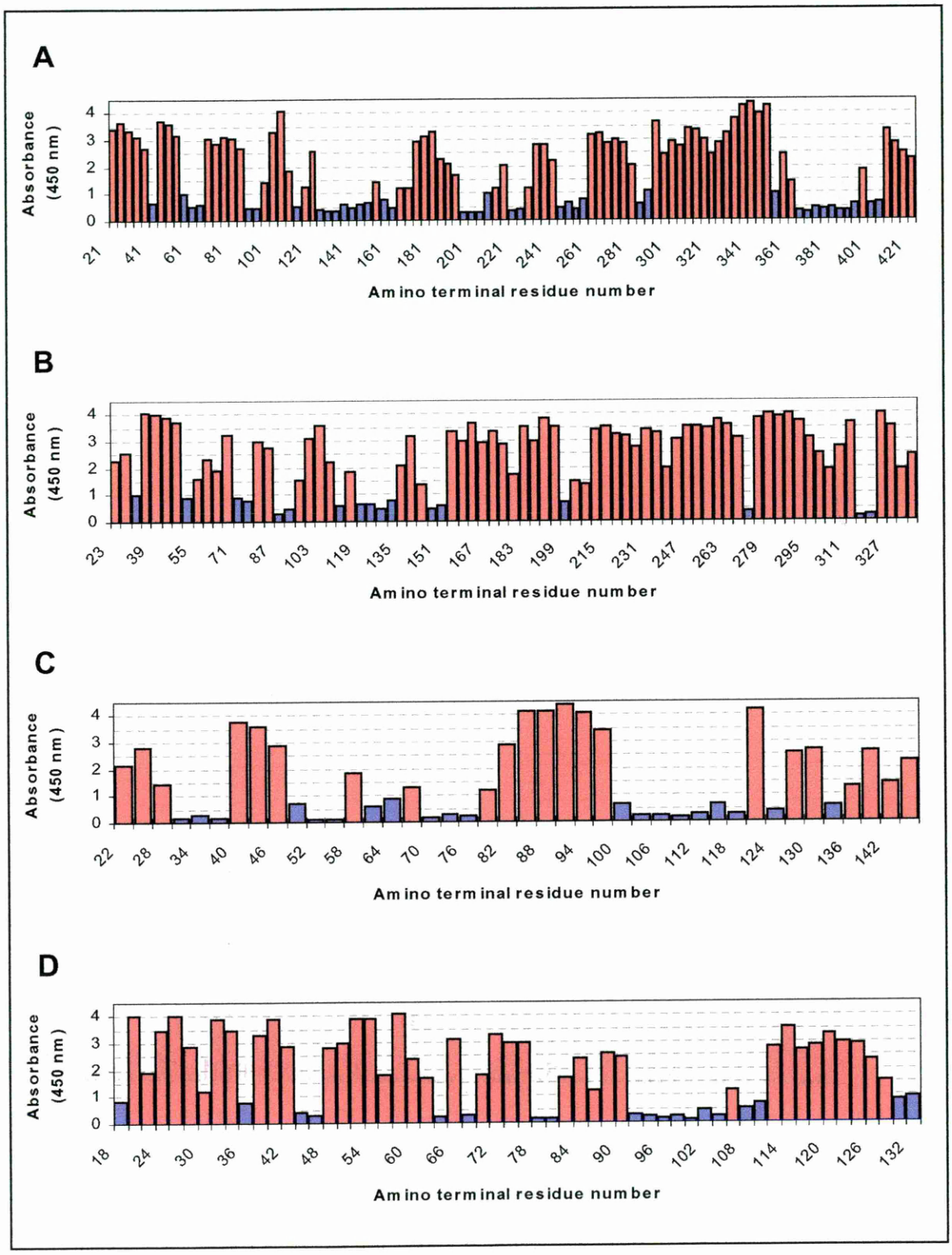


Figure 44. Epitope scans produced by a strongly reacting serum specimen from a patient with syphilis.

A; Absorbances of the set of 14-mer overlapping peptides covering TpN47. B; Absorbances of the set of 14-mer overlapping peptides covering TpN44.5. C; Absorbances of the set of 12-mer overlapping peptides covering TpN17. D; Absorbances of the set of 10-mer peptides covering TpN15.



Affinity purified antibody preparations specific for each of the recombinant proteins were prepared from a pool of syphilitic serum specimens. The affinity purified antibodies produced dramatically different epitope scans to those of the original serum pool from which they were derived. Fig 45 compares the epitope scans of TpN47 before and after affinity purification. Compared to the original material, the affinity purified antibodies show a large increase in the response of the reactive peptides and, proportionately, a much smaller increase in the absorbances of the least reactive peptides. This produces much more clearly defined reactive regions than is seen with the original serum pool. A similar overall picture is seen in the epitope scans of the other antigens using the appropriately purified antibody preparation. The results of TpN44.5 are shown in Fig 46, those of TpN17 in Fig 47, while Fig 48 features the scans of TpN15. It is interesting to note the similarity of results produced by the affinity purified antibody preparations to those of the most reactive individual sera tested as shown in Fig 44.

Figure 45. Epitope scans of TpN47 using a syphilitic serum pool before and after affinity purification.

Absorbances of the set of 14-mer peptides covering the sequence of TpN47. The dark red and dark blue bars represents the syphilitic serum pool and the light red and light blue represents it after affinity purification. Red bars represent peptides greater than the cut-off value.

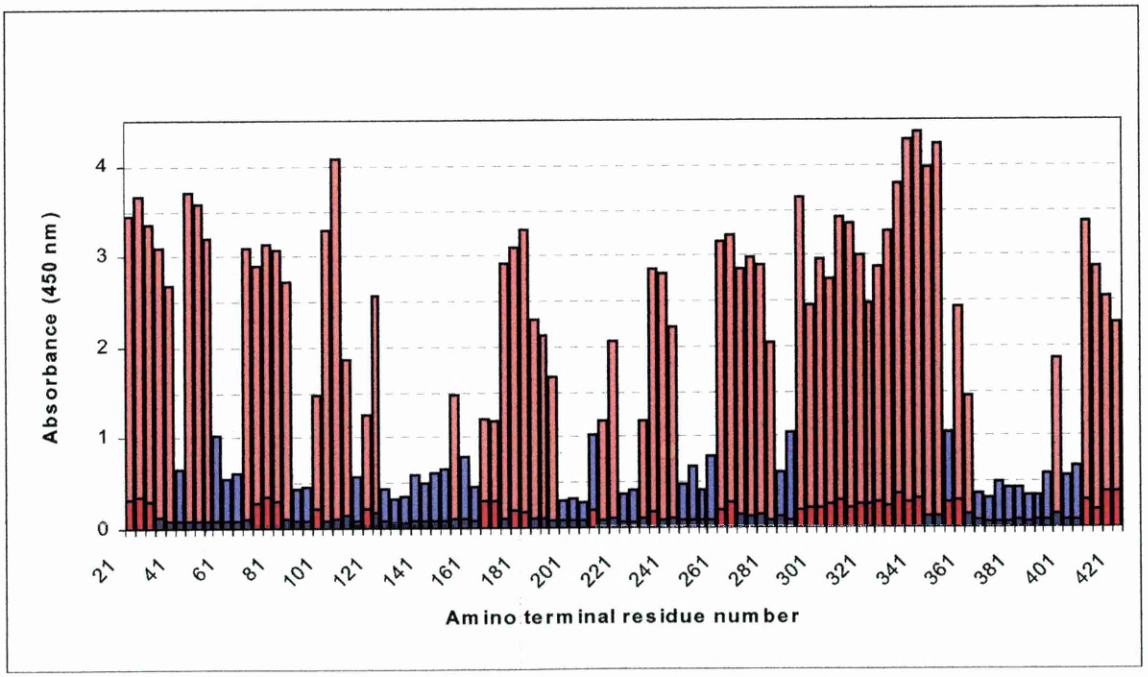


Figure 46. Epitope scans of TpN44.5 using a syphilitic serum pool before and after affinity purification.

Absorbances of the set of 14-mer peptides covering the sequence of TpN44.5. The dark red and dark blue bars represents the syphilitic serum pool and the light red and light blue represents it after affinity purification. Red bars represent peptides greater than the cut-off value.

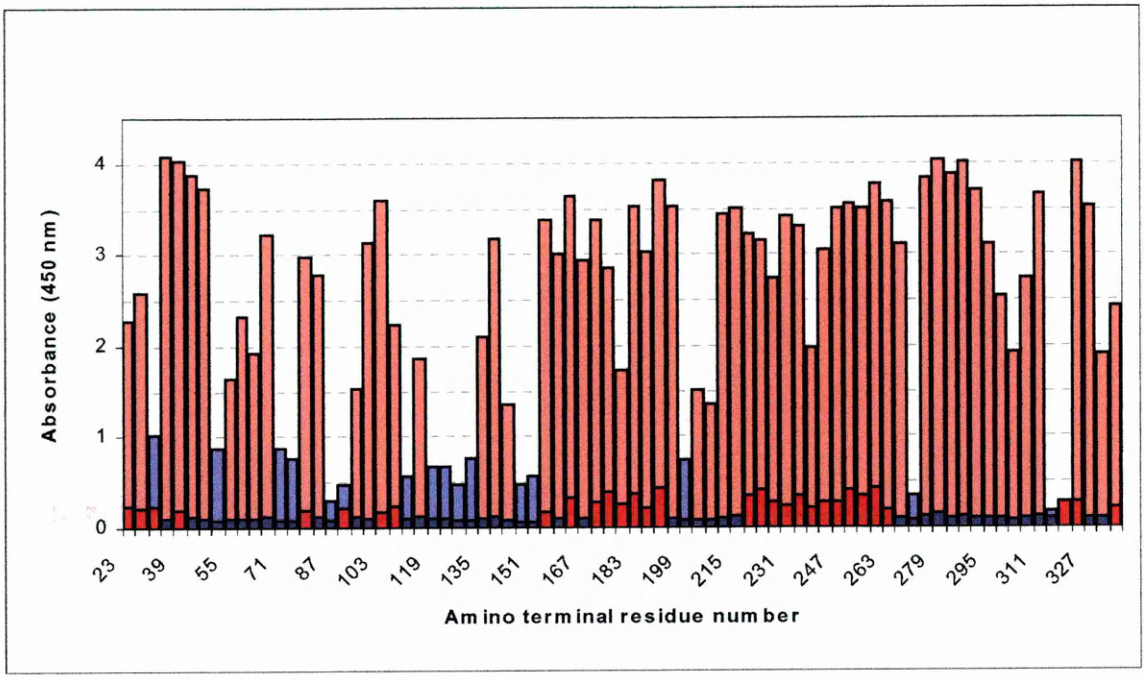


Figure 47. Epitope scans of TpN17 using a syphilitic serum pool before and after affinity purification.

Absorbances of the set of 12-mer peptides covering the sequence of TpN17. The dark red and dark blue bars represents the syphilitic serum pool and the light red and light blue represents it after affinity purification. Red bars represent peptides greater than the cut-off value.

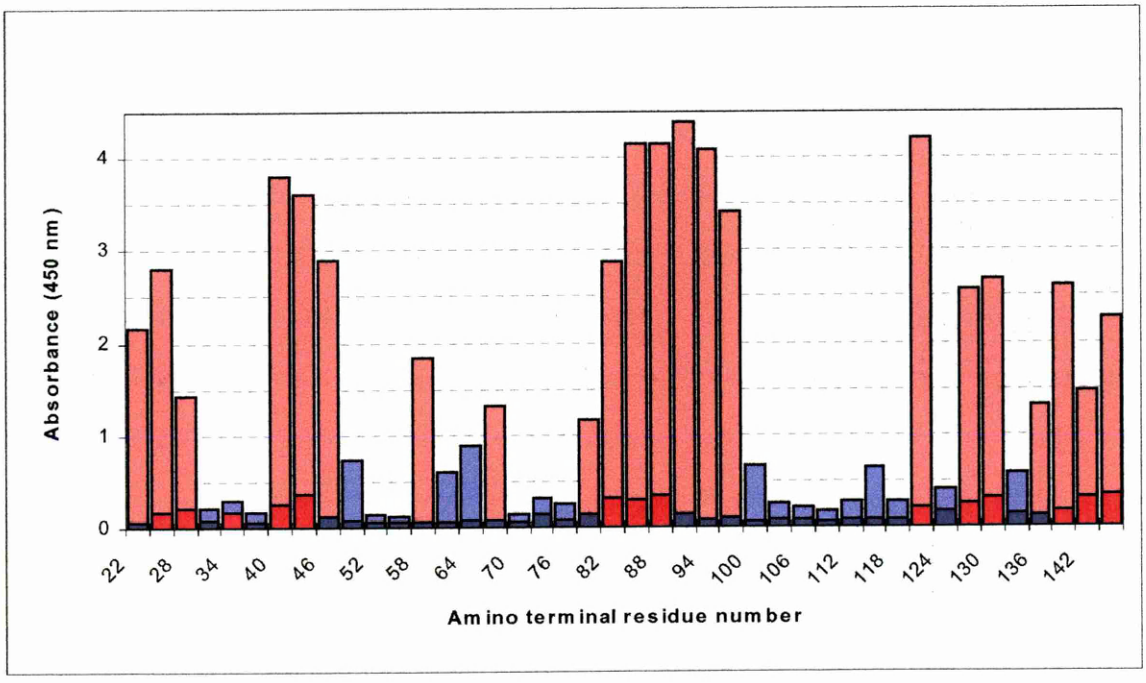
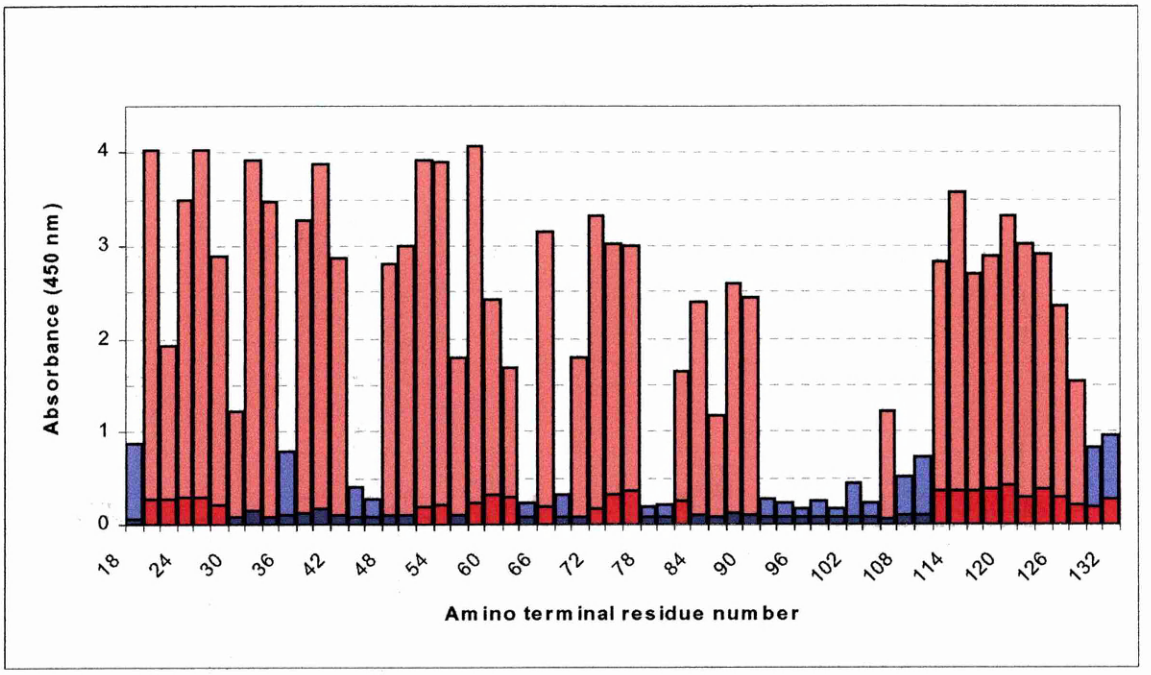


Figure 48. Epitope scans of TpN15 using a syphilitic serum pool before and after affinity purification.

Absorbances of the set of 10-mer peptides covering the sequence of TpN15. The dark red and dark blue bars represents the syphilitic serum pool and the light red and light blue represents it after affinity purification. Red bars represent peptides greater than the cut-off value.



Dramatic variations in the responses to individual peptides among the various serum specimens was noted. The epitope scans produced a complicated picture of multiple reactivities across the protein with no obvious consensus. In an attempt to analyse the results, frequency profiles were constructed based on the number of sera reacting with each peptide and also based on the total absorbance for each peptide. Neither approach proved useful in interpreting the epitope scans. The most successful approach was to plot the reactive regions of each serum epitope scan, which were defined as a sequence of at least two consecutive reactive peptides. In order to identify consensus reactive sequences, plots were constructed of all the reactve regions identified by the various syphilitic sera. Fig 49 summarises the data for the peptides covering TpN47. Fig 50, Fig 51, and Fig 52 show similar data summaries for TpN44.5, TpN17, and TpN15, respectively. Significant immunoreactive regions could be identified that were reactive with the majority of sera tested. TpN47 shows such six such regions spanning amino acid start positions, A; 21-33, B; 69-85, C; 165-185, D; 261-277, E; 293-361 and F; 409-421. TpN44.5 and TpN17 each show two such regions spanning amino acid start positions, A; 171-195 and B; 219-263, and A; 79-91 and B; 139-145, respectively. TpN15 shows four regions spanning amino acid start positions A; 20-28, B; 52-56, C; 72-76 and D; 112-132.

Figure 49. Summary of the reactive regions of TpN47.

Sequences of two or more reactive peptides in the epitope scans of 22 serum specimens from patients with syphilis (black), a syphilitic serum pool (blue) and an affinity purified anti-TpN47 antibody preparation (red).

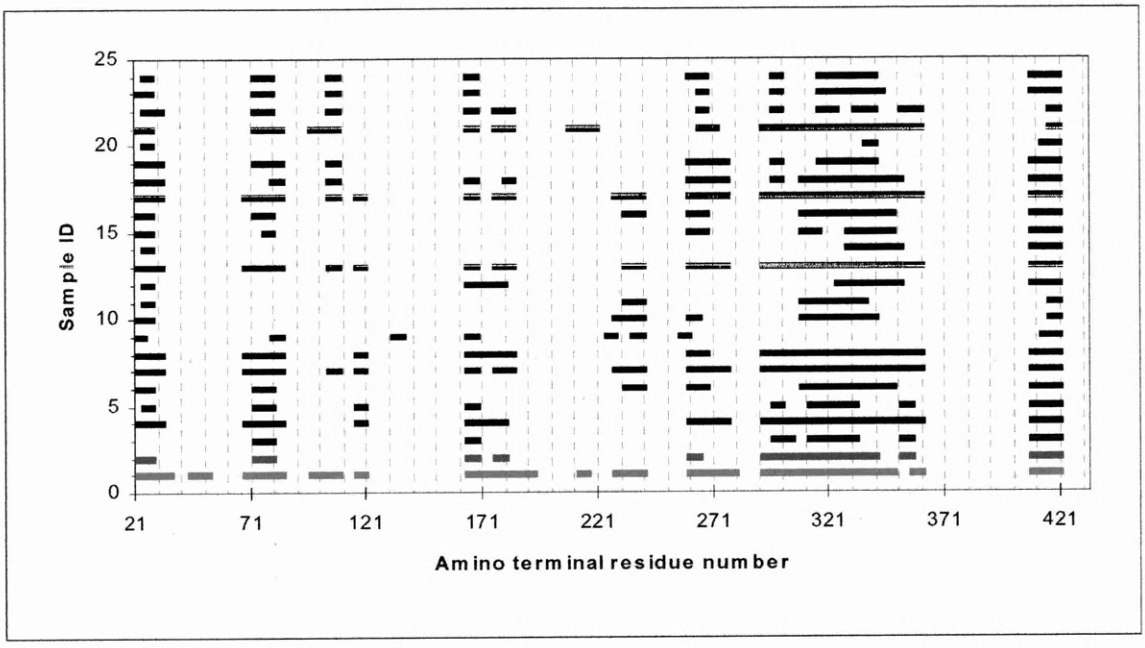


Figure 50. Summary of the reactive regions of TpN44.5.

Sequences of two or more reactive peptides in the epitope scans of 22 serum specimens from patients with syphilis (black), a syphilitic serum pool (blue) and an affinity purified anti-TpN44.5 antibody preparation (red).

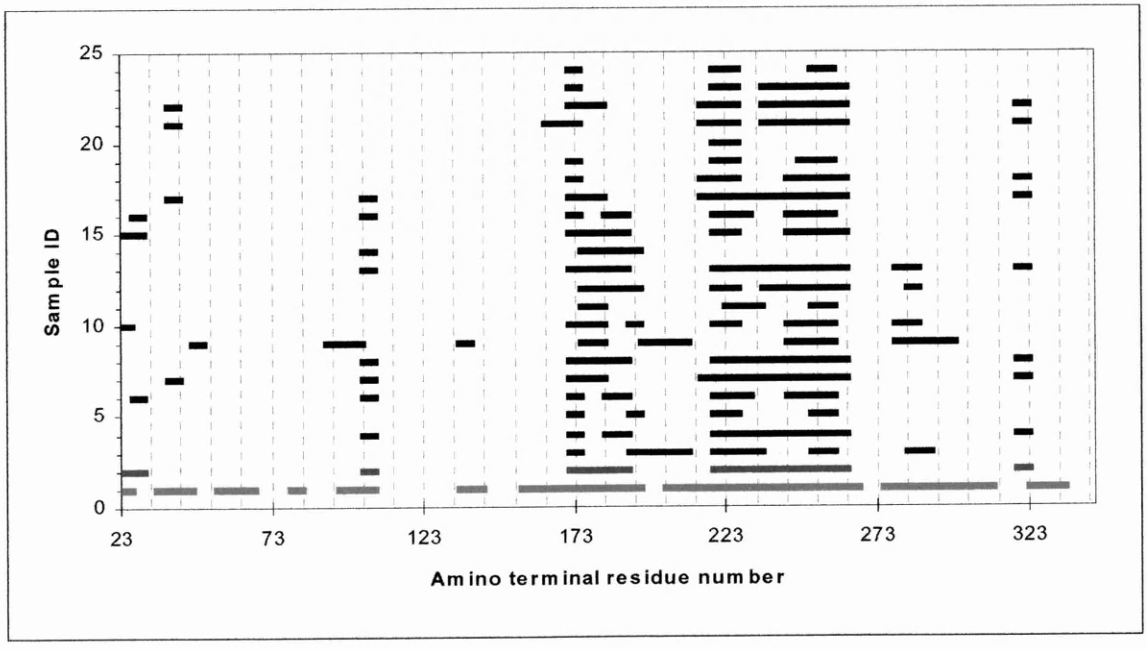


Figure 51. Summary of the reactive regions of TpN17.

Sequences of two or more reactive peptides in the epitope scans of 22 serum specimens from patients with syphilis (black), a syphilitic serum pool (blue) and an affinity purified anti-TpN17 antibody preparation (red).

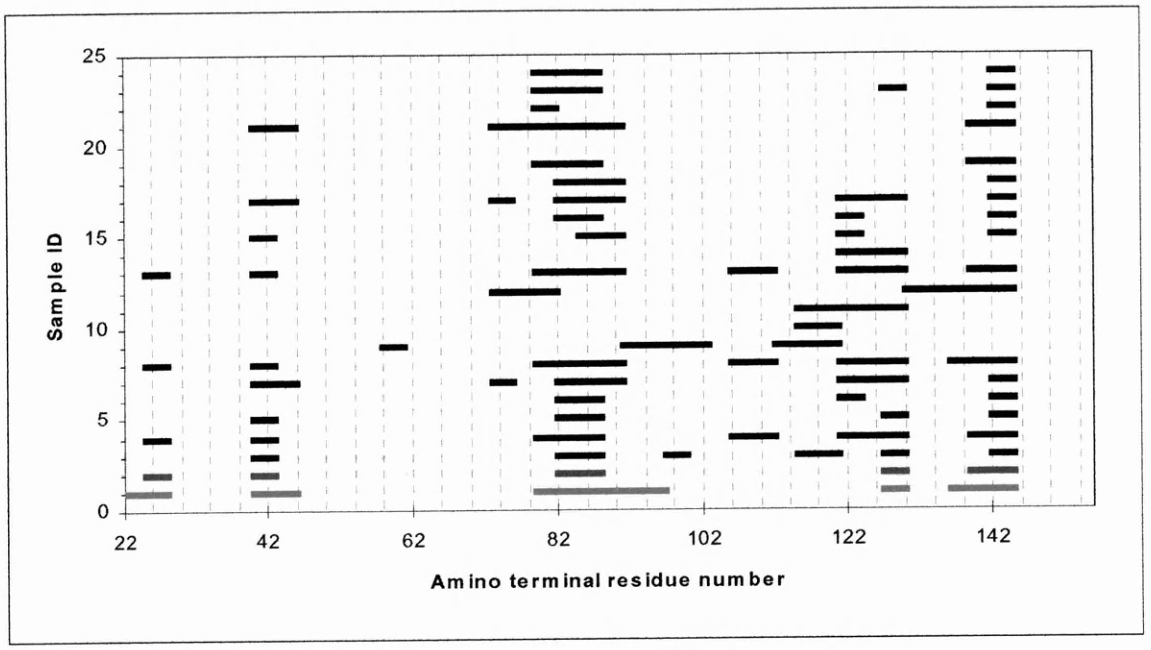
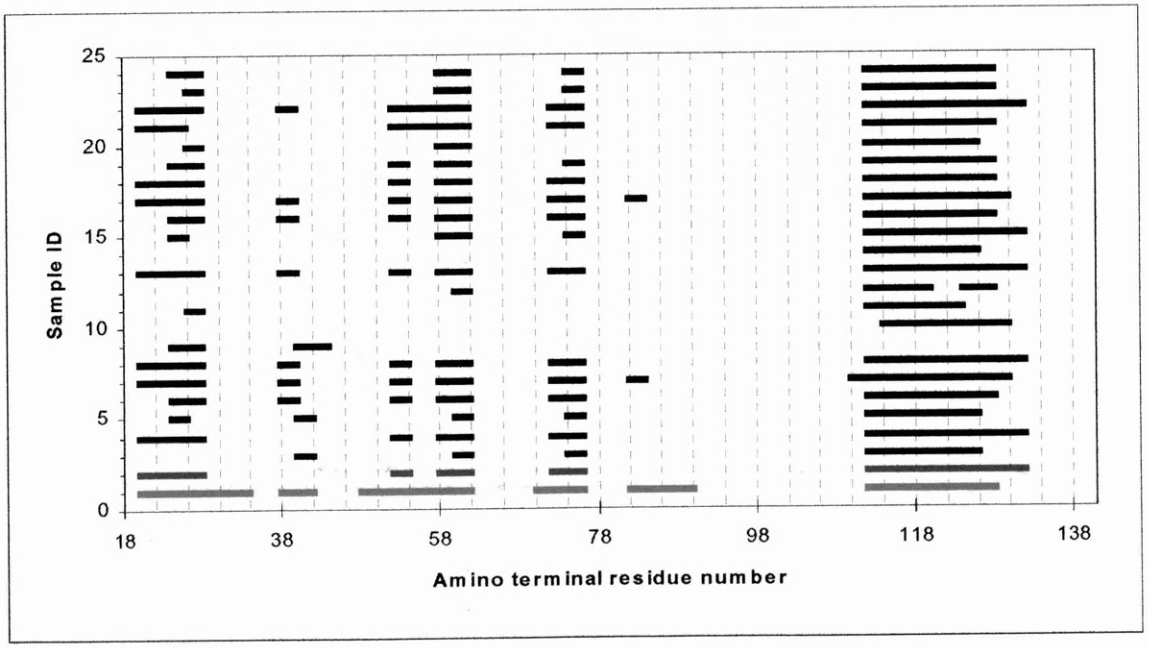


Figure 52. Summary of the reactive regions of TpN15.

Sequences of two or more reactive peptides in the epitope scans of 22 serum specimens from patients with syphilis (black), a syphilitic serum pool (blue) and an affinity purified anti-TpN15 antibody preparation (red).



Discussion

The results of synthetic peptide epitope mapping experiments very much depend on the accurate synthesis of the peptides. This was monitored by the presence of bromophenol blue in the reaction mixture to detect any unreacted amino groups. After each round of the synthesis process the absence of bromophenol blue staining of the pins indicated that the amino acid had been successfully added to the sequence. No staining was detected after any of the coupling steps, therefore the peptides were assumed to be correctly synthesised, and should correspond to the sequences of the experimental plan. The synthesis procedure was also controlled using functional activity. Control peptides (PLAQ and GLAQ) were synthesised in parallel with the test peptides. The reactivity of in-process control peptides, with a specific monoclonal antibody, compared well to that of similar peptides supplied by the manufacturer, suggesting the sequences were not only synthesised correctly, but also functionally active. The nature of the peptide synthesis and the format used necessitated a sequential testing programme. Therefore, it was necessary to confirm the successful regeneration of the peptides and determine the consistency of the testing program, if comparative analysis was to be performed. Periodic testing of the peptides after regeneration with conjugate only, failed to detect residual antibody on any occasion. Therefore, the regeneration procedure effectively and reliably removed all traces of bound antibodies between testing runs. The repeated cycles of testing and regeneration could lead to deterioration of the peptides. The virtually identical results of the same serum specimen tested at the beginning and end of the testing programme suggests that there was no detectable loss of activity of the peptides over the course of the experiment. The similarity of the absorbances demonstrates not only the lack of deterioration, but also the reproducibility of the testing protocol making comparison of the results of different sera possible.

Even though there was considerable variation in the response to individual peptides and weak reactions were relatively common, an overall consensus of the reactive regions was found which suggests the process is fairly robust. The weak nature of some responses is probably due to the low specific activity of polyclonal human sera because only a small proportion of the IgG present will be disease specific. Affinity purification will not only purify out the specific antibodies, but has the tendency to favour the selection of high affinity variants. This has the effect of increasing the specific activity of the sample and also eliminating weak or non-specific reactivities. These effects

are shown clearly by the affinity purified antibody preparations in the strength and specificity of their reactions with the peptides. The responses were dramatically different from the weak indistinct results of the raw serum pool from which the affinity purified antibodies were derived. They produced much stronger responses with more clearly defined sequences of peptides, and low background reactions. The immunoreactive regions identified by the affinity purified antibodies agree with the consensus reactivities of the other specimens. They also define some regions only detected by a minority of specimens. The use of affinity purification in epitope mapping studies is an innovative approach and seems particularly applicable to situations where only weak, indistinct serological reactions are present.

The literature contains a huge number of reports of epitope mapping studies of a large number of antigens from a range of different microorganisms. Review of the extensive literature in this field reveals only three reports describing epitope mapping studies of polypeptides of *T.pallidum*, and no studies of closely related organisms. The major lipoproteins of *T.pallidum* have no known function and no homologues have been identified in other organisms, so comparisons with the results of similar studies on related proteins are not possible. This means that the analogous literature available for discussion is relatively restricted.

Baughn *et al.* (1996a) have comprehensively investigated the B-cell determinants of TpN47 using syphilitic rabbit sera and IgM fractions of human sera. These investigators also found that there were dramatic variations in the response with individual peptides. By constructing plots based on the numbers of sera reacting with each peptide they identified a series of ten immunoreactive regions, none of which agree with the regions identified in this study. In fact the two sets of results seem mutually exclusive of each other, the reactive regions of Baughn *et al.* coinciding virtually exactly with the non-reactive sequences identified here. This is difficult to reconcile solely on the basis of differences in the peptide length and overlap used as the peptides used by Baughn *et al.* were similar to those used in this study. However, the nature of the sera tested may have some influence on the results obtained. The rabbit antisera used by Baughn *et al.* will not only have a much higher specific activity than immune human sera used in the present study, but the immune response might be directed towards a different set of species-specific epitopes, accounting for the different results seen in the two studies. Similarly, the results obtained by Baughn *et al.* using human IgM specimens may indicate that the IgM response is directed towards fundamentally

different epitopes to the IgG response examined in this study. No studies could be found in the literature that investigated whether the IgM and IgG antibody responses are directed towards different epitopes. Further testing of the peptide with IgM fractions and hyperimmunised rabbit antisera would be required to determine if the differences between the results reported by Baughn *et al.* and those described in the present study are due to differences in the peptides used, or due to the type of specimen tested.

In another study, Baughn *et al.* (1996b) have epitope-mapped TpN15 using immunised rabbit serum and identified seven discrete regions of reactivity: I, 10-17; II, 20-27; III, 49-60; IV, 69-79; V, 84-92; VI, 107-113; VII, 115-128. The region I identified by Baughn *et al.* occurs completely within the signal sequence of the protein. The set of peptides used in the present study did not include the signal sequence, and so no conclusions can be drawn concerning region I. Regions A, B, and C identified in the study reported here matched regions II, III, and VI, respectively, to within a few amino acids one way or the other. Region D can be considered to match regions VI and VII, which are virtually contiguous producing a larger region covering residues 107-128. Region V matches a sequence that is only detected by the affinity purified antibody and not by any of the individual specimens tested. The hyperimmunised rabbit serum used by these researchers will be much more avid, and of a higher titre than immune serum from a patient with syphilis, and possess many of the characteristics of an affinity purified preparation. Region V is only detected by antibodies produced after artificially enhanced immunisation or purified to increase their potency, which suggests that this region is probably weakly immunogenic. Generally, these two studies agree quite closely on which sequences within TpN15 are immunoreactive, even though they use different antibody sources.

The antigenic structure of TpN44.5 has recently been investigated (Antoni *et al.*, 1996) using an alternative approach. A series of ten overlapping peptides, 35-40 amino acids long covering the entire sequence were synthesised. They were coated on to microplates and tested by enzyme immunoassay against serum specimens from patients with syphilis. These researchers found the most reactive peptide to be the one covering the N-terminal portion of the sequence between residue numbers 23-60. They found a second region of lesser reactivity at the C-terminal end of the protein between residues 250-345. This contrasts with the results found here where the main reactivity is in a central region of the protein between amino acids 171 and 263. The likely cause of

this discrepancy is in the differences in the experimental protocols used. The enzyme immunoassay method requires the peptides to be bound to the microplate wells. This brings with it problems of differential binding that will affect the level of response in the assay. So, a weak reaction might not be due to low reactivity with a peptide but actually due to poor binding of the peptide to the plastic surface. This highlights another limitation of the enzyme immunoassay procedure, the need to use relatively long peptides to allow them to be bound to plastic. Short peptides bind very poorly to polystyrene surfaces. The strategy used by Antoni *et al.* (1996) selected the size of the peptides, the position of the junctions between peptides, and their overlaps on the basis of a hydrophobicity plot. This could impose a predetermined outcome to the experiment due to the position of the junctions between the chosen peptides. However, this approach does have some advantages. Using large fragments of the protein could mean that conformational epitopes are present that would be absent when small peptides are used. This could account for the differences in the reactions observed by Antoni *et al.* compared to those seen in the study reported here, and could mean that a significant antibody response to TpN44.5 is directed towards a conformational epitope contained within the first 38 amino acids of the mature protein sequence. This epitope would not be identified using the small peptides featured in the present study as they exhibit little or no secondary structure. Clearly the strategy used can profoundly affect the results generated.

A huge literature of epitope mapping studies exists, examining the antigens of a wide range of viruses, bacteria and other microorganisms, as well those of cell constituent proteins and enzymes. However, there is a preponderance of reports describing the epitopes of viral capsid proteins, and the target epitopes of monoclonal antibodies. There are relatively few reports on bacterial membrane proteins and none at all on other lipoproteins, so it is difficult to place the work reported here in context. In the studies examined, a commonly reported pattern of reaction is to see several reactive regions across a protein each containing multiple epitopes (Bolwell *et al.*, 1989.; Middeldorp and Melsen, 1988.; Okahaski *et al.*, 1993.; Strynadka *et al.*, 1988.; Zhong *et al.*, 1990.). This is a similar general pattern to that seen in the study reported here.

A report (Geysen *et al.*, 1988), by some of the inventors of solid-phase peptide epitope mapping and the holders of key patents, suggests that it is not uncommon when using human sera for multiple weak reactivities to be reported and the poor signal to background ratios are not improved by increasing the concentration of the test sera. Their interpretation of this type of data is that the

serum lacks a major population of antibodies directed to a linear epitope of the antigen. Many or all of the small peaks present are believed to be due to segments of discontinuous determinants. This conclusion is drawn from the similarity of the signals to those obtained when testing a monoclonal antibody known to recognise a discontinuous epitope. Further they suggest, based on unpublished epitope mapping studies with monoclonal antibodies, that approximately 5-10% of all antibodies directed to native antigens bind to linear epitopes (Geysen *et al.*, 1987). This suggests that a large proportion of the antibody responses seen in the study reported here are possibly directed towards discontinuous epitopes and so are poorly defined by small synthetic peptides.

In view of the large literature it is surprising how few of the sequences identified by epitope mapping studies have been used to develop successful diagnostic tests. Volkmer-Engbert *et al.* (1994) and Wout *et al.* (1993) are two examples of epitope mapping studies that produced successful peptide-based antigens for the diagnosis of HIV and EBV infections, respectively. It is of note that they are both diagnostically significant viral outer envelope proteins and show a distinctly different reaction pattern to the many of the other studies, and also the study reported here. These proteins show only a few reactive regions with most of the reactivity confined to a single relatively short sequence. This pattern of antigenicity allows a relatively short sequence to capture most of the antibody response and so lends itself to the use of synthetic peptide-based antigen. However, when the antibody response is directed to determinants spread across the whole protein sequence, as in the study reported here, a large number of peptides would be required to capture enough antibody to be diagnostically effective. This makes the use of synthetic peptides impractical and lends itself to the use of recombinant proteins. It is for this reason, and the success of the recombinant protein approach, that the sequences identified here were not investigated any further.

Summary

Epitope mapping with overlapping synthetic peptides is an efficient way to identify the linear antigenic determinants defined by a particular serum or monoclonal antibody. However, a large proportion of the total epitopes present, the discontinuous or conformational epitopes, are inaccessible by this method. The antibody responses were not found to be directed towards clearly defined sequences of linear epitopes in a background of low responses, as is seen in the previously cited examples of diagnostically effective peptides. The technique identified multiple responses

across the entire sequence of the proteins, however some consensus antigenic sequences were identified. This overall complex picture suggests that a high proportion of the diagnostically significant antibody response is probably directed to discontinuous conformational epitopes.

Chapter 9

Phage display epitope mapping

Introduction

Filamentous bacteriophages such as M13, fl, and fd have three to five copies of the capsid protein III (cpIII) located at one end of the phage particle. This protein is essential for proper phage assembly and for adsorption to the F pilus of male strains of *E.coli*. When small DNA fragments are inserted between the leader sequence and the mature coding sequence of the gene coding for cpIII, the progeny phage carry the corresponding protein sequence as an N-terminal fusion with the cpIII protein (Parmley and Smith, 1988). Such fusions do not affect the function of the cpIII protein, so the phage are able to propagate normally. In the technique known as phage display, a library of different peptides or proteins are displayed on the surface of the phage particles. Phage expressing particular motifs, such as antibody binding properties, can be enriched in the population using a form of affinity selection known as biopanning. The library is first incubated with a target molecule bound to a solid-phase, then the unbound phage are washed away. The bound phage are eluted from the solid-phase by acidification and amplified, thus increasing the number of phage carrying binding motifs in the population. The enriched population is then biopanned again against a fresh solid phase coated with the target material. Usually, three to four rounds of this enrichment process are completed before the primary structure of the binding motifs are deduced by nucleotide sequencing of individual clones.

Phage display can be utilised to locate antigenic epitopes. Fragments of DNA that encode portions of the protein antigen are fused to the gene encoding capsid protein III. Phage can then be tested with the antibody to determine which displayed fragments react with the antibody. This has been done successfully for the HIV *gag* gene (Tsunetsugu-Yokota *et al.*, 1991) and more recently for the outer capsid protein VP5 of the bluetongue virus (Wang *et al.*, 1995). This is the basic concept explored in the work described in the study reported here. The simplest form of display, as utilised in the fUSE2 vector used in this study, is achieved by insertion of the DNA fragments directly into the gene coding for the cpIII protein, this means that all copies of cpIII carry the resulting fusion. Some sequences, usually large proteins like antibody fragments, are poorly displayed using this type of display because of interference with the function of cpIII by the presence of the large fusion protein. This has been overcome using a different type of vector system. The DNA fragments are clone into a phagemid vector. When bacterial cells harbouring these phagemids are infected with M13 helper phage, which carry a full complement of capsid-encoding genes but are defective in

replication, the secreted phage particles carry the phagemid genome and a mixture of wild-type and fusion cpIIIs. The presence of wild-type cpIII ensures the phage particles can adequately interact with the F pilus and propagate normally. This type of display is exemplified by the SurfZAP vector system used in the study reported here.

In the work described here, the objective was to produce random gene fragment phage display libraries which expressed sequences of the four lipoprotein antigens of *T.pallidum*, TpN47, TpN44.5, TpN17, and TpN15. In accomplishing this objective two different vector systems and two different methods of preparing the gene fragments were employed. Random gene fragments were prepared by a nested PCR technique using a primer pair which rebuilt the portion of the cpIII leader sequence missing from the vector and terminated in a series of random bases. The rationale was that the random bases of the primers would anneal at arbitrary points along the gene creating the random fragments. Then a second set of primer complementary to the 5' ends of the first primer pair would be used, after removal of excess primers from the first reaction, to amplify the reaction products of the first PCR. These DNA fragments were cloned into the SurfZAP vector system to produce a set of phagemid libraries. The second method used was simpler and involved producing DNA fragments of the lipoprotein genes by digestion with DNase 1. Following size selection and modification of the fragment termini, they were cloned into the *Bgl*II site of the fUSE2 vector, thus producing a library of phage particles displaying random fragments of the lipoprotein antigens. These libraries were biopanned with antibodies specific for *T.pallidum* and the binding motifs of individual clones identified by nucleotide sequencing.

Epitope mapping by phage display was employed because it has the potential to supply complementary information to that of the previous studies using synthetic peptides. Synthetic peptides epitope mapping studies are limited to identifying linear or sequential epitopes due to the restricted size of the peptides used. Phage display can express a larger range of sizes of protein fragments, therefore it allows an array of different motifs to be displayed from short linear epitopes to longer sequences with the potential to express significant conformational structure. The objective of the phage display work described here was to identify antigenic sequences within the lipoprotein antigens of *T.pallidum* preferentially bound by specific antibodies regardless of size. The type of fragments selected by the biopanning procedure would give valuable information on the nature of the antibody response to *T.pallidum*. If predominantly short sequences were selected this would

suggest that the significant antibody responses were principally directed towards linear epitopes. However, if long sequences were selected then conformational epitopes represent the most significant targets of the antibody response. Alternatively, long sequences might contain multiple epitopes allowing multipoint attachment of the protein, thereby enhancing retention by the solid-phase. Finally, any sequences identified would be of great interest as antigens for use in serological tests.

Results

PCR synthesised random gene fragment library

Random gene fragments were generated by a nested PCR technique, using random primers to amplify segments of the target genes, which were then cloned into the SurfZAP phage display vector system (Stratagene). Separate gene fragment libraries were constructed for TpN47, TpN44.5, TpN17 and TpN15. Table 3 shows the sizes of the primary libraries before amplification, estimated by lambda plaque titration. After amplification each library consisted of a total of 10^{11} pfu per library, at a concentration of $2x10^9$ pfu per mL.

Table 3. Number of phage successfully packaged for each of the primary libraries.

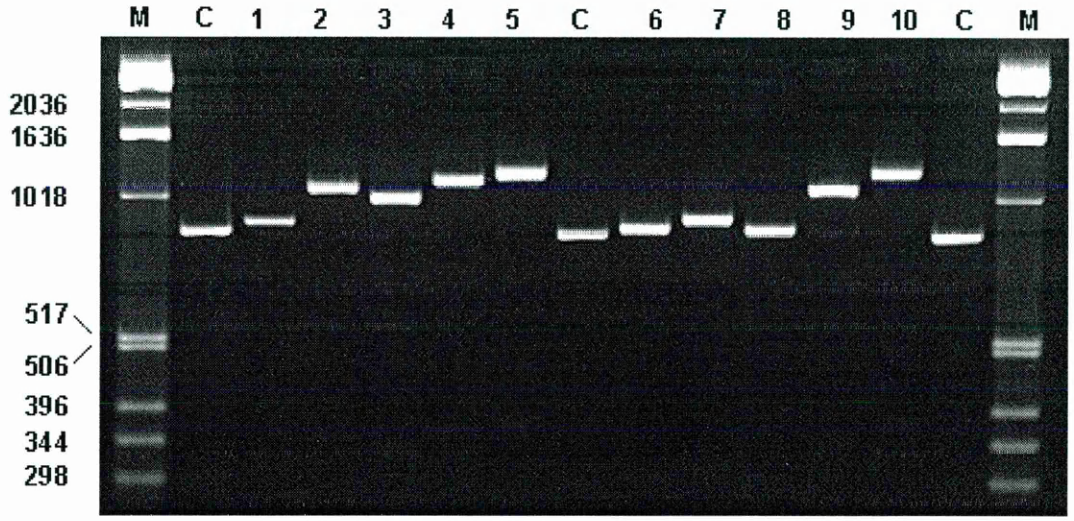
Target gene	Library designation	Total number of pfu packaged	Number of pfu in primary library
*tpn*47	SZ47	$8.2x10^5$	$8.1x10^5$
*tpn*44.5	SZ44	$3.7x10^5$	$3.6x10^5$
*tpn*17	SZ17	$3.4x10^5$	$3.3x10^5$
*tpn*15	SZ15	$4.1x10^5$	$4x10^5$
No insert control	-	$1x10^4$	-

10 random plaques were randomly selected from each of the primary libraries and the size of their inserts determined by PCR amplifying across the cloning site using standard T3 and T7 promoter primers. Fig 53 shows the results of the plaques selected from the SZ47 library. The theoretical size of the amplified DNA from the vector only is 882bp. The randomly selected plaques produced

amplified DNA segments with a range of sizes and all larger than the vector only controls. Similar amplifications of randomly selected plaques from the other three libraries also showed a range of insert sizes, all larger than the vector only controls (data not shown).

Figure 53. Characterisation of SZ47 library complexity.

PCR amplified inserts of 10 random plaques from the SZ47 primary library separated on a 2.5% agarose gel. Lane M; 1kb DNA ladder (bp are indicated). Lane C; SurfZAP vector only. Lane 1-10; 10 random plaques.



The four libraries were biopanned against a solid phase coated with purified IgG from a pool of serum from patients with high titre syphilis antibodies. The number of phage recovered from the solid phase was estimated after each round of biopanning by phage titring. There was no increase in the number of phage eluted from the solid phase in successive rounds of biopanning. Random clones arbitrarily selected from the enriched libraries failed to produce a signal when tested in the M13 immunoassay.

In another attempt at biopan enrichment, the four libraries were biopanned against solid-phases coated with affinity purified antibody of the same specificity as the library. After each successive round of biopanning there was an increase in the number of phage recovered from the solid phases. After three rounds, individual colonies were randomly selected from each of the enriched libraries and the inserts analysed by PCR, M13 immunoassay and DNA sequencing.

PCR screening

The insert contained in each clone was PCR amplified using T3 and T7 promoter primers that anneal to sequences in the vector flanking the cloning site. Fig 54 shows the amplified inserts in the clones selected from the SZ47 and SZ44 separated by agarose gel electrophoresis. The size of the inserts were estimated by interpolation against the DNA standards. The SZ47 library clones produced a range of insert sizes: clones 1, 3, 5, 6, 7 and 8 contain an insert 45 bp long, clones 2 and 9 contain a 90 bp insert, while clone 10 contains a much larger insert approximately 222 bp long. Clones 4 and 11 give bands of the same size as the vector only control, so probably contain no inserts. The clones selected from the SZ44 library produces two types of inserts. Clones 12, 15, 18 and 20 produce bands of the same size as the vector only control, therefore probably contain no inserts, and clones 13, 14, 16, 17, 19, 21 and 22 contain a 342 bp insert.

Figure 54. Insert sizes of colonies from biopan enriched SZ47 and SZ44 libraries.

PCR amplified inserts of randomly selected SZ47 and SZ44 colonies separated on a 2.5% agarose gel. Lane M; 1kb DNA ladder (bp are indicated). Lane C; fUSE2 vector only. Lane W; No DNA PCR control. Lane 1-11; 11 randomly selected SZ47 colonies after enrichment by biopanning with affinity purified anti-TpN47 antibodies. Lane 12-22; 11 randomly selected SZ44 colonies after enrichment by biopanning with affinity purified anti-TpN44.5 antibodies.

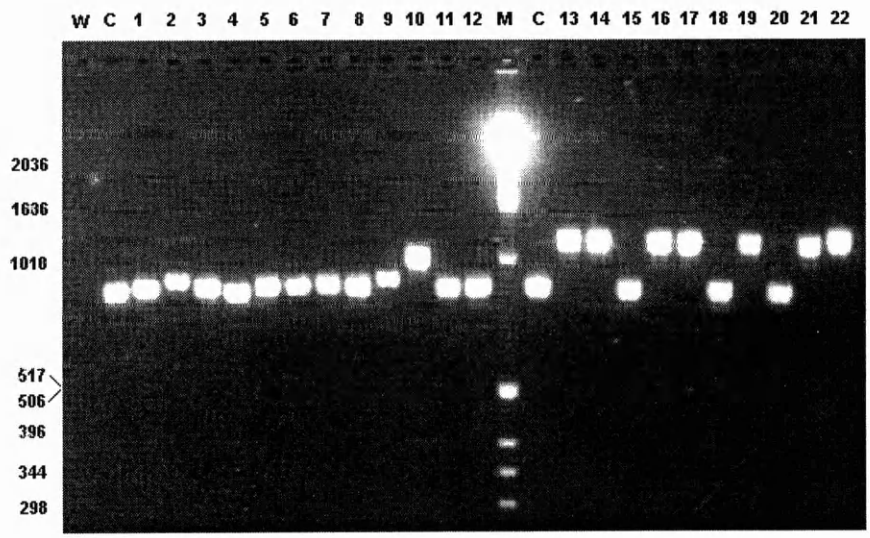
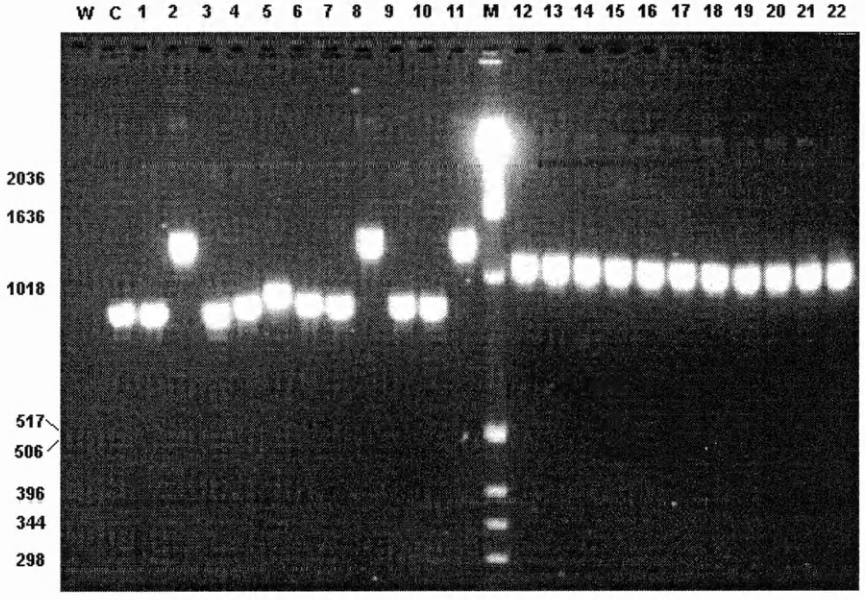


Fig 55 shows the amplified inserts of clones selected from the SZ17 and SZ15 libraries. Again, the size of the inserts were estimated by interpolation against the DNA standards. The SZ17 library produced a range of insert sizes. Clones 2, 8, and 11 contained an insert approximately 393 bp long. Clones 1 and 3 contain no insert. The insert in clone 5 is approximately 84 bp long and the

insert in clone 6 is 45 bp long. Clones 4, 7, 9 and 10 appear to contain a 33 bp insert. The clones selected from the enriched SZ15 library all contain an insert approximately 255 bp long.

Figure 55. Insert sizes of colonies from biopan enriched SZ17 and SZ15 libraries.

PCR amplified inserts of randomly selected SZ17 and SZ15 colonies separated on a 2.5% agarose gel. Lane M; 1kb DNA ladder (bp are indicated). Lane C; fUSE2 vector only. Lane W; No DNA PCR control. Lane 1-11; 11 randomly selected SZ17 colonies after enrichment by biopanning with affinity purified anti-TpN17 antibodies. Lane 12-22; 11 randomly selected SZ15 colonies after enrichment by biopanning with affinity purified anti-TpN15 antibodies.



M13 immunoassay

Recombinant phage particles were prepared from the clones analysed by PCR and tested in an M13 immunoassay. Fig 56 shows the absorbances of the 11 clones, previously tested by PCR, selected from the enriched SZ47 library. All the clones produce a strong signal with the anti-M13 coated wells, but low absorbances with the test wells coated with anti-TpN47 and control wells coated with human IgG. The VCSM13 helper phage produced the expected response with the anti-M13 coated well and the diluent control gives low absorbances with all wells. Fig 57 and Fig 58 show the results of the clones selected from the SZ44 and SZ17 libraries, respectively. They show a similar pattern of reactions to those already described for the SZ47 clones, strong responses with the anti-M13 coated wells and little response to the affinity purified antibody coated wells and normal IgG control wells. The results of the clones from the SZ15 library show a different pattern of reactions. All samples show a strong response to the anti-M13 coated wells and a weaker response

to the anti-TpN15 coated wells that is distinctly greater than that seen with the normal IgG coated wells. Again, the VCSM13 and diluent controls give the expected responses.

Figure 56. M13 immunoassay results of clones selected from enriched SZ47 library.

Absorbances of 11 randomly selected SZ47 clones after enrichment by three rounds of biopanning against affinity purified anti-TpN47 antibodies.

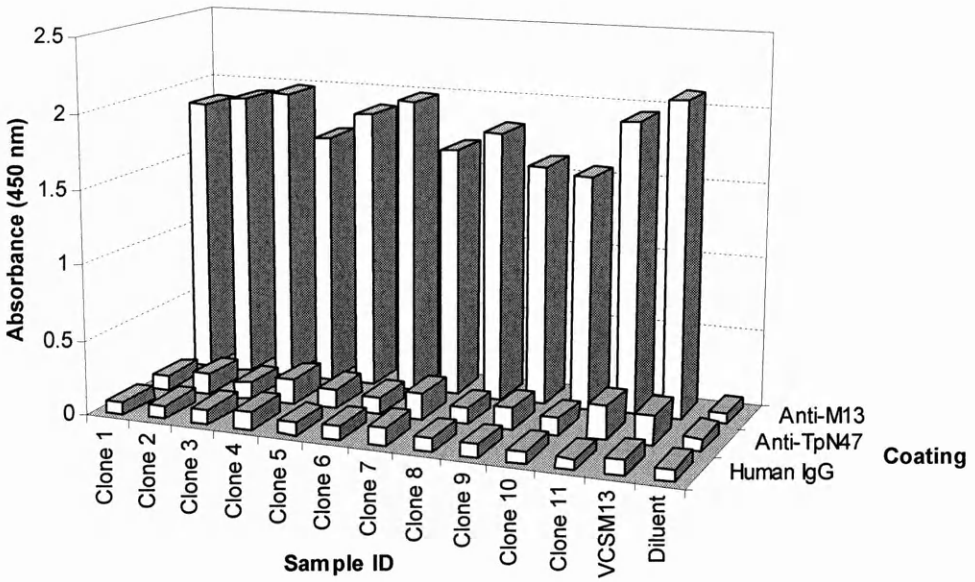


Figure 57. M13 immunoassay results of clones selected from enriched SZ44 library.

Absorbances of 11 randomly selected SZ44 clones after enrichment by three rounds of biopanning against affinity purified anti-TpN44.5 antibodies.

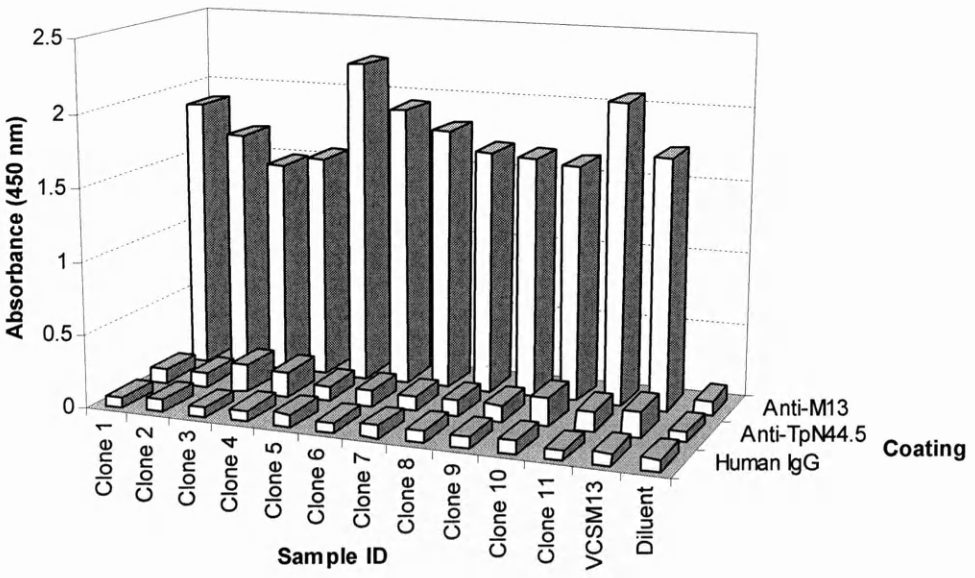


Figure 58. M13 immunoassay results of clones selected from enriched SZ17 library.

Absorbances of 11 randomly selected SZ17 clones after enrichment by three rounds of biopanning against affinity purified anti-TpN17 antibodies.

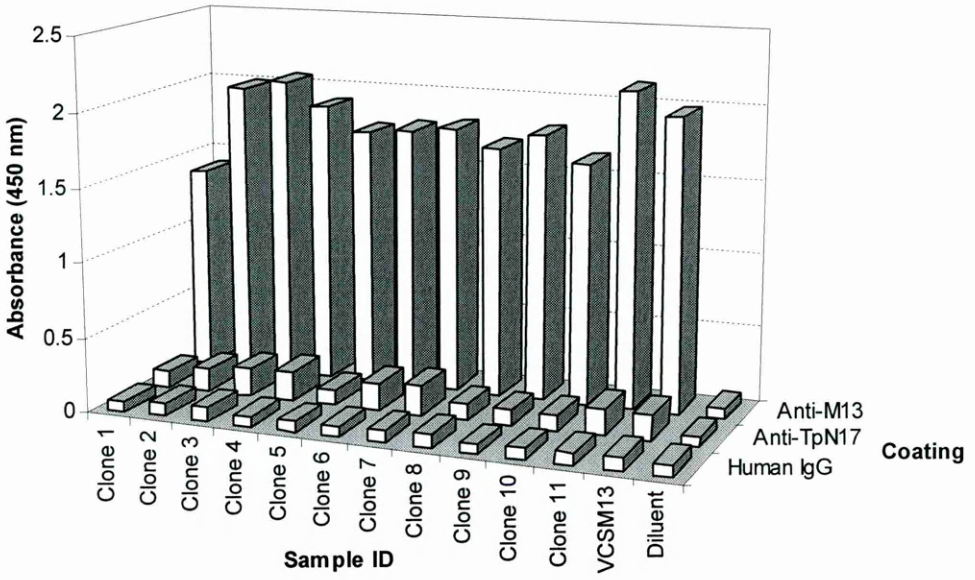
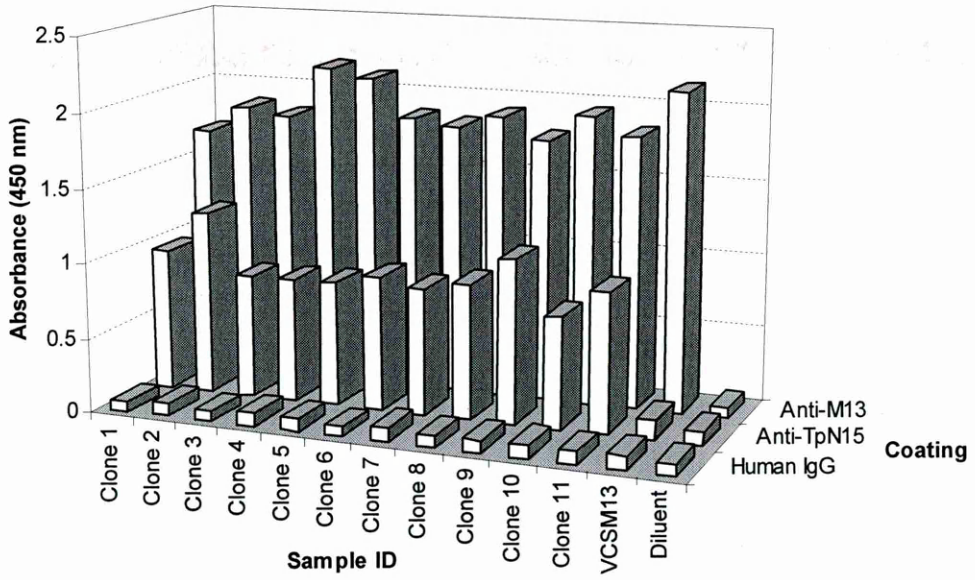


Figure 59. M13 immunoassay results of clones selected from enriched SZ15 library.

Absorbances of 11 randomly selected SZ15 clones after enrichment by three rounds of biopanning against affinity purified anti-TpN15 antibodies.



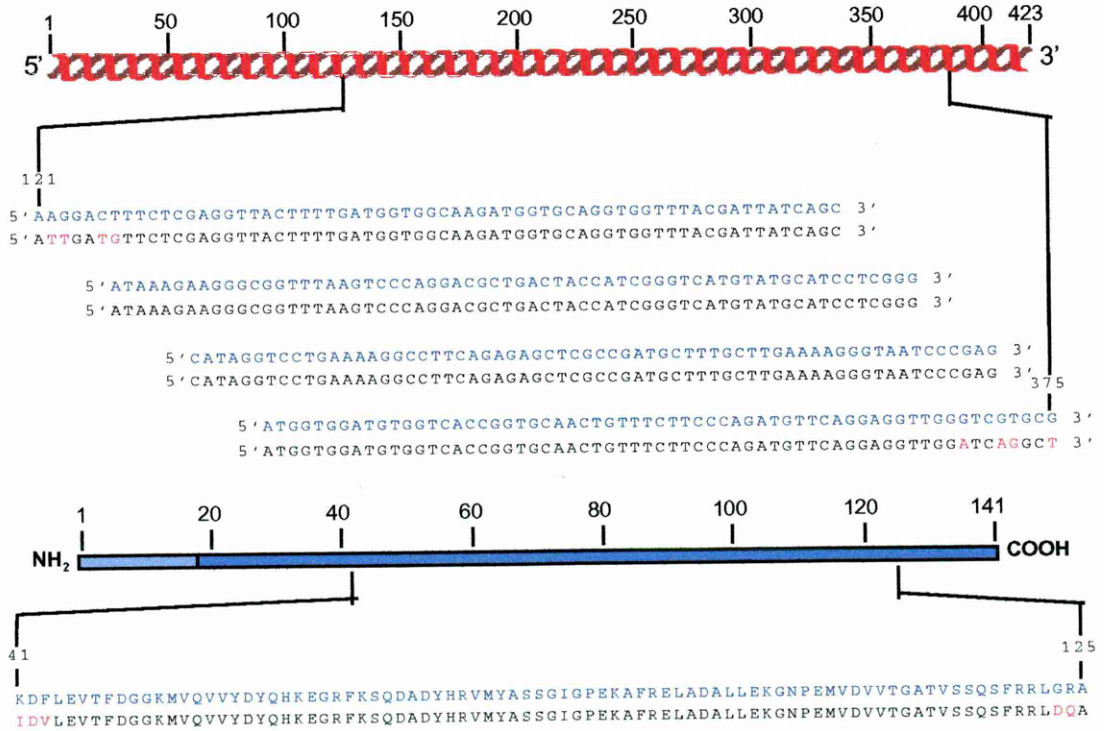
DNA sequences

Several clones were sequenced to identify the inserted DNA sequences. Clones 2, 5 and 10 from the SZ47 library, clones 21 and 22 from the SZ44 library, and clones 2, 5, 6, and 7 from the SZ17 library were sequenced. The sequences were compared with the known database sequences of the *T.pallidum* lipoproteins. None of the inserts showed homology with the *T.pallidum* lipoproteins at either a nucleotide or amino acid level.

Clones 12 and 19 from the SZ15 library were sequenced and both found to contain the same 255 bp insert. This insert was found to have homology to a sequence within the TpN15 lipoprotein of *T.pallidum* between residue 121 and 375. Fig 60 shows the alignment of the DNA sequence of the insert (black) with the known database sequence of that region of *tpn*15 (blue) and highlights the discrepant nucleotides in red. The deduced amino acid sequences associated with the DNA sequences are also shown for comparison.

Figure 60. DNA sequence of clone 12 from the enriched SZ15 library.

DNA and deduced amino acid sequences of the insert of clone 12 biopanned from the SZ15 library using affinity purified anti-TpN15 antibodies. Database sequence is shown in blue and the insert sequence in black, with non homologous residues in red. The amino acid sequence is deduced from the DNA sequence.



DNase1 digested random gene fragment library

A single random gene fragment library was constructed by digesting the four major lipoprotein genes with DNase I, and cloning the resulting fragments into fUSE2 phage display vector. The number of recombinant phage produced under different ligation conditions was estimated by phage titring and the results are shown in Table 4. Electroporation of the replicative form (RF) DNA produced the large number of transformants expected. The ligation reactions at different vector:insert ratios all produced similar numbers of transformants, but less than uncut RF DNA produced. These reactions produced significantly more transformants than when no insert was present in the reaction. The four reactions containing the recombinant phage were pooled giving a primary library of around 10^6 phage, which was amplified to give a stock of 10^{12} cfu/mL.

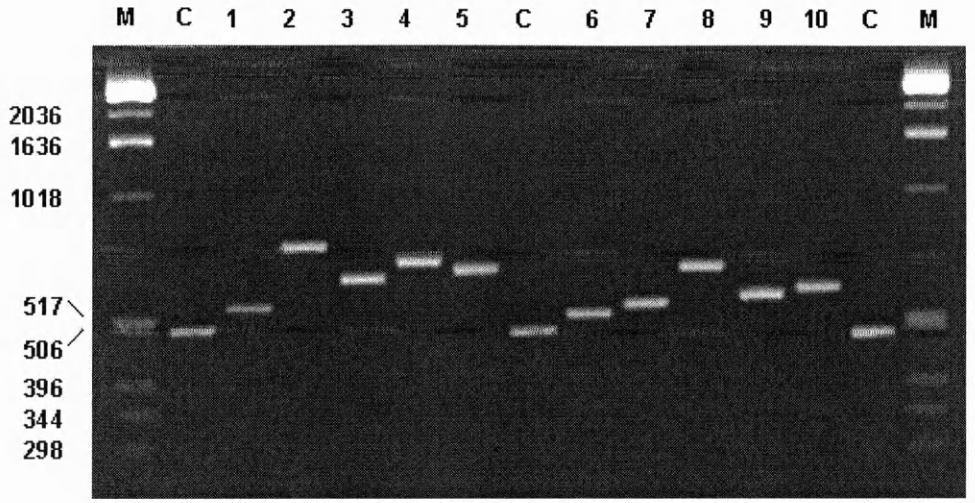
Table 4. Number of recombinant phage produced under different ligation conditions.

Ligation reaction	No. of phage / µg DNA
RF DNA	$2x10^7$
Vector:Insert ratio = 1:0	$1x10^4$
Vector:Insert ratio = 1:2	$0.8x10^5$
Vector:Insert ratio = 1:3	$2.3x10^5$
Vector:Insert ratio = 1:4	$1.8x10^5$
Vector:Insert ratio = 1:5	$5.1x10^5$
MC1061 *E.coli* cells only	none detected

The library was characterised by PCR amplifying across the cloned insert using primers (cpIII-S, cpIII-T) that annealed to vector encoded sequences. 10 random colonies were selected, the RF DNA isolated and used as template for the PCR. The amplified DNA was separated by electrophoresis on a 2.5% agarose gel which is shown in Fig 61. Amplification across the cloning site theoretically produces a fragment of 496 bp, which is confirmed by the DNA in lane C. All ten of the clones selected contained an insert of various sizes, which covered the range of sizes (50 - 350 bp) in the original DNase1 digestion.

Figure 61. Characterisation of fUSE2 library.

Amplified inserts of 10 random plaques from the fUSE2 primary library separated on a 2.5% agarose gel. Lane M; 1kb DNA ladder (bp are indicated). Lane C; fUSE2 vector only. Lane 1-10; 10 random colonies.



The four libraries were biopanned against a solid phase coated with purified IgG from a pool of serum from patients with high titre syphilis antibodies.

The library was biopanned against a solid phase coated with purified IgG from a pool of serum from patients with high titre syphilis antibodies. The number of phage recovered from the solid phase was estimated after each round of biopanning by colony titring. There was no increase in the number of phage eluted from the solid phase in successive rounds of biopanning. Randomly selected clones from the enriched libraries failed to produce a signal when tested in the M13 immunoassay.

The library was biopanned against four different solid-phases. The solid phases were prepared by separately coating affinity purified antibodies to TpN47, TpN44.5, TpN17 and TpN15. After each successive round of biopanning there was an increase in the number of phage recovered from the solid phases. After three rounds, individual colonies were arbitrarily selected from each of the enriched libraries and the inserts were characterised by PCR amplification, M13 immunoassay, and DNA sequencing.

PCR screening

12 colonies were randomly selected after biopan enrichment, RF DNA isolated and the insert PCR amplified as previously described. Fig 62 shows the DNA separated on a 2.5% agarose gel. The sizes of the inserts were estimated by interpolation against the DNA standards. Eleven of the twelve

from the library biopanned with anti-TpN47 contain a 141 bp insert, except clone 8 that contains no insert. The inserts from the library biopan enriched against anti-TpN44.5 show two types of insert. Clones 18 and 24 contain an insert approximately 153 bp long and the rest have a smaller 87 bp insert.

Figure 62. fUSE2 insert sizes after enrichment with anti-TpN47 or anti-TpN44.5 antibodies.

PCR amplified inserts of randomly selected fUSE2 colonies separated on a 2.5% agarose gel. Lane M; 1kb DNA ladder (bp are indicated). Lane C; fUSE2 vector only. Lane W; No DNA PCR control. Lane 1-12; 12 random colonies selected after enrichment by biopanning with affinity purified anti-TpN47 antibodies. Lane 13-24; 12 random colonies selected after enrichment by biopanning with affinity purified anti-TpN44.5 antibodies.

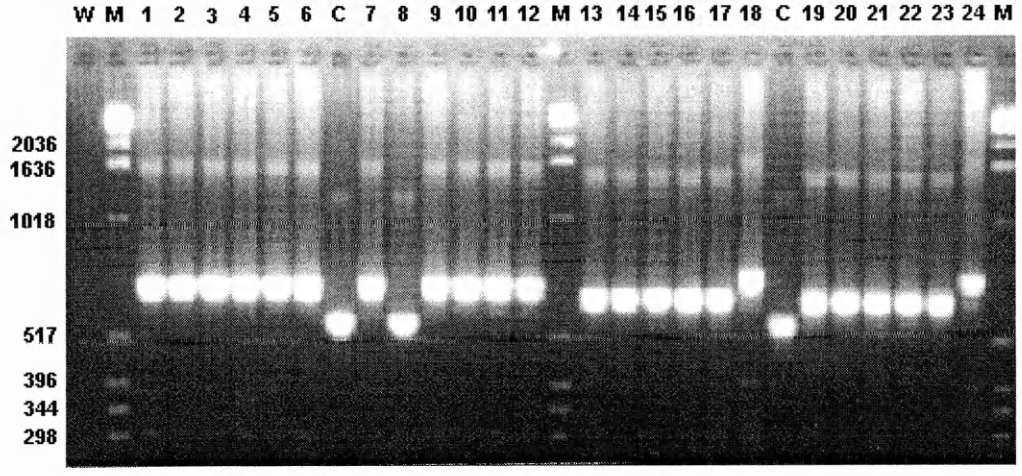


Figure 63. fUSE2 insert sizes after enrichment with anti-TpN17 or anti-TpN15 antibodies.

PCR amplified inserts of randomly selected fUSE2 colonies separated on a 2.5% agarose gel. Lane M; 1kb DNA ladder (bp are indicated). Lane C; fUSE2 vector only. Lane W; No DNA PCR control. Lane 1-12; 12 random colonies selected after enrichment by biopanning with affinity purified anti-TpN17 antibodies. Lane 13-24; 12 random colonies selected after enrichment by biopanning with affinity purified anti-TpN15 antibodies.

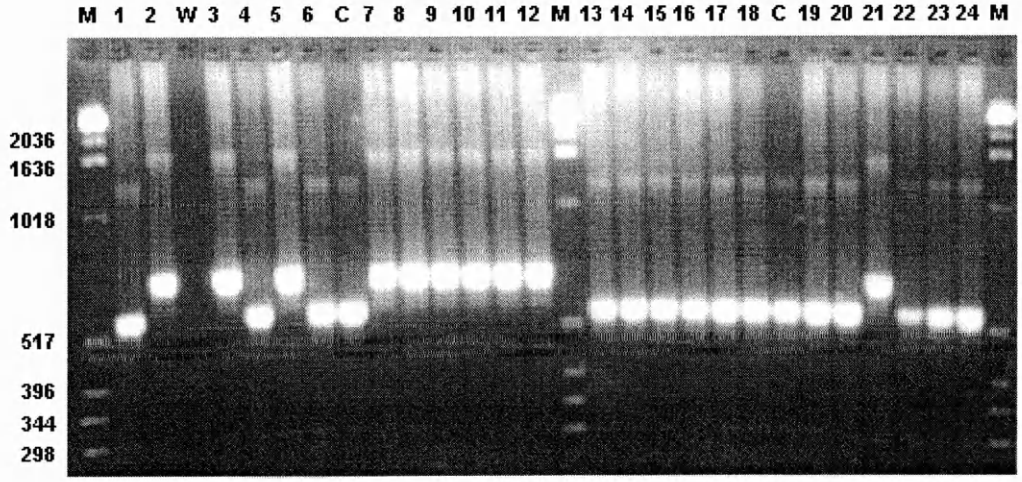


Fig 63 shows the results of similar amplifications of the insert in clones selected from libraries enriched against anti-TpN17 and anti-TpN15 solid phases. Nine out of the twelve phage picked from the library biopanned against anti-TpN17 have a 123 bp insert. The remaining three clones isolated, 1, 4, and 6, contain no insert. Eleven of the twelve phage isolated from the library biopanned with anti-TpN15 have no insert. Only clone 21 contains an insert which seems to be 78 bp long.

M13 immunoassay

Samples of each of the recombinant phage particles were tested in an M13 immunoassay. All phage samples produced a strong response with the anti-M13 antibody coated wells and gave low absorbances with the negative control wells coated with normal human IgG. All assays gave the expected results with the VCSM13 helper phage and diluent controls. Fig 64 shows the absorbances of the phage selected after enrichment against affinity purified anti-TpN47. Eleven of the twelve clones gave a response with the affinity purified antibody coated wells, only clone 8 shows a response of a similar level to that seen with the negative control wells. Fig 65 shows the results for the clones selected after biopanning with affinity purified anti-TpN44.5 antibodies. All phage samples show a good response with the affinity purified antibody coated wells, although there is some variation in the level of the responses. Fig 66 shows the results of the clones selected after enrichment against the affinity purified anti-TpN17. Clones 1, 4, and 6 give low absorbances similar to those of the negative controls, while the other nine clones produce a good response with the affinity purified antibody coated wells. Fig 67 shows the results of the clones selected after biopanning against affinity purified anti-TpN15. All 12 clones produce responses similar to those of the negative control wells, although there is a strong response with the anti-M13 coated wells.

Figure 64. M13 immunoassay results of anti-TpN47 enriched fUSE2 clones.

Absorbances of 12 randomly selected fUSE2 clones from the third round of biopanning against an affinity purified anti-TpN47 antibody coated solid phase.

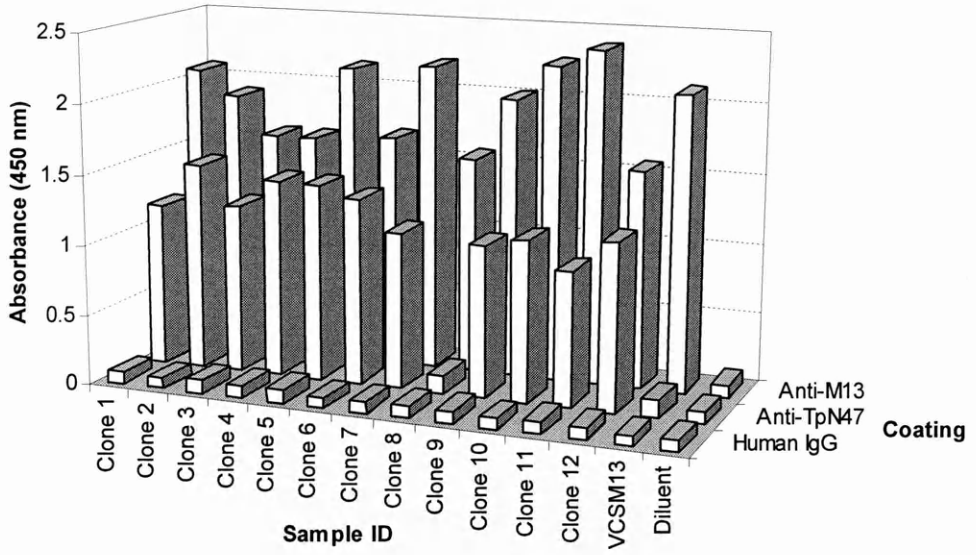


Figure 65. M13 immunoassay results of anti-TpN44.5 enriched fUSE2 clones.

Absorbances of 12 randomly selected fUSE2 clones from the third round of biopanning against an affinity purified anti-TpN44.5 antibody coated solid phase.

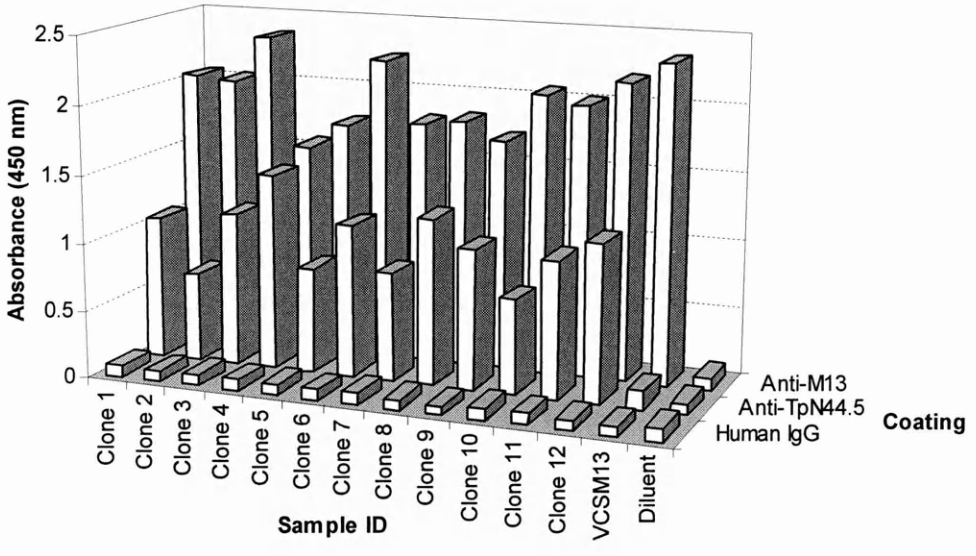


Figure 66. M13 immunoassay results of anti-TpN17 enriched fUSE2 clones.

Absorbances of 12 randomly selected fUSE2 clones from the third round of biopanning against an affinity purified anti-TpN17 antibody coated solid phase.

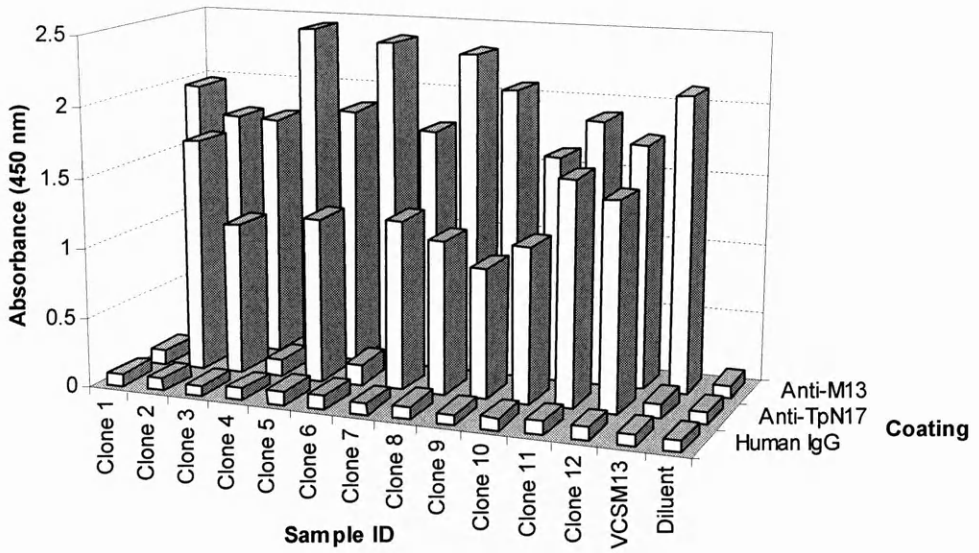
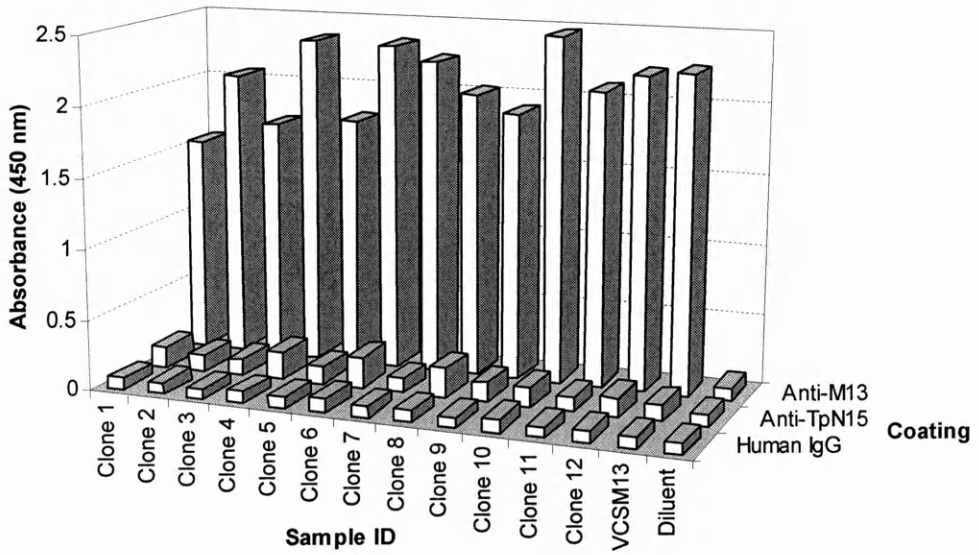


Figure 67. M13 immunoassay results of anti-TpN15 enriched fUSE2 clones.

Absorbances of 12 randomly selected fUSE2 clones from the third round of biopanning against an affinity purified anti-TpN15 antibody coated solid phase.

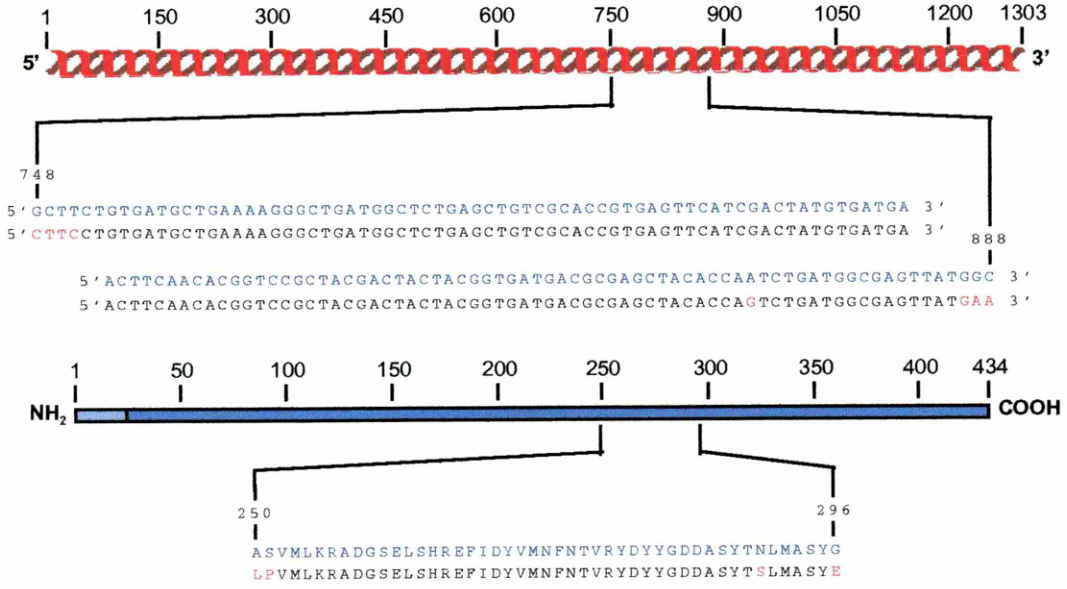


DNA sequencing

Several clones from each enriched library were sequenced to identify the inserted DNA sequences. The clones selected from the fUSE2 library biopanned against anti-TpN47 showed a single size of insert, so clones 4 and 7 were sequenced and found to contain the same 141 bp insert. This insert was found to have sequence homology to the *tpn*47 gene of *T.pallidum* between base pairs 748 and 888, which corresponds to residues 250 and 296 of the amino acid sequence. Figure 68 shows the alignment of the DNA sequence with the database sequence, and also shows the deduced amino acid sequences for comparison. The database sequence is shown in blue and the insert sequence in black, with non homologous residues highlighted in red.

Figure 68. DNA sequence of clone 4 from the anti-TpN47 enriched fUSE2 library.

DNA and deduced amino acid sequences of the insert of clone 4 biopanned from the fUSE2 library using affinity purified anti-TpN47 antibodies. Database sequence is shown in blue and the insert sequence in black, with non homologous residues in red. The amino acid sequence is deduced from the DNA sequence.

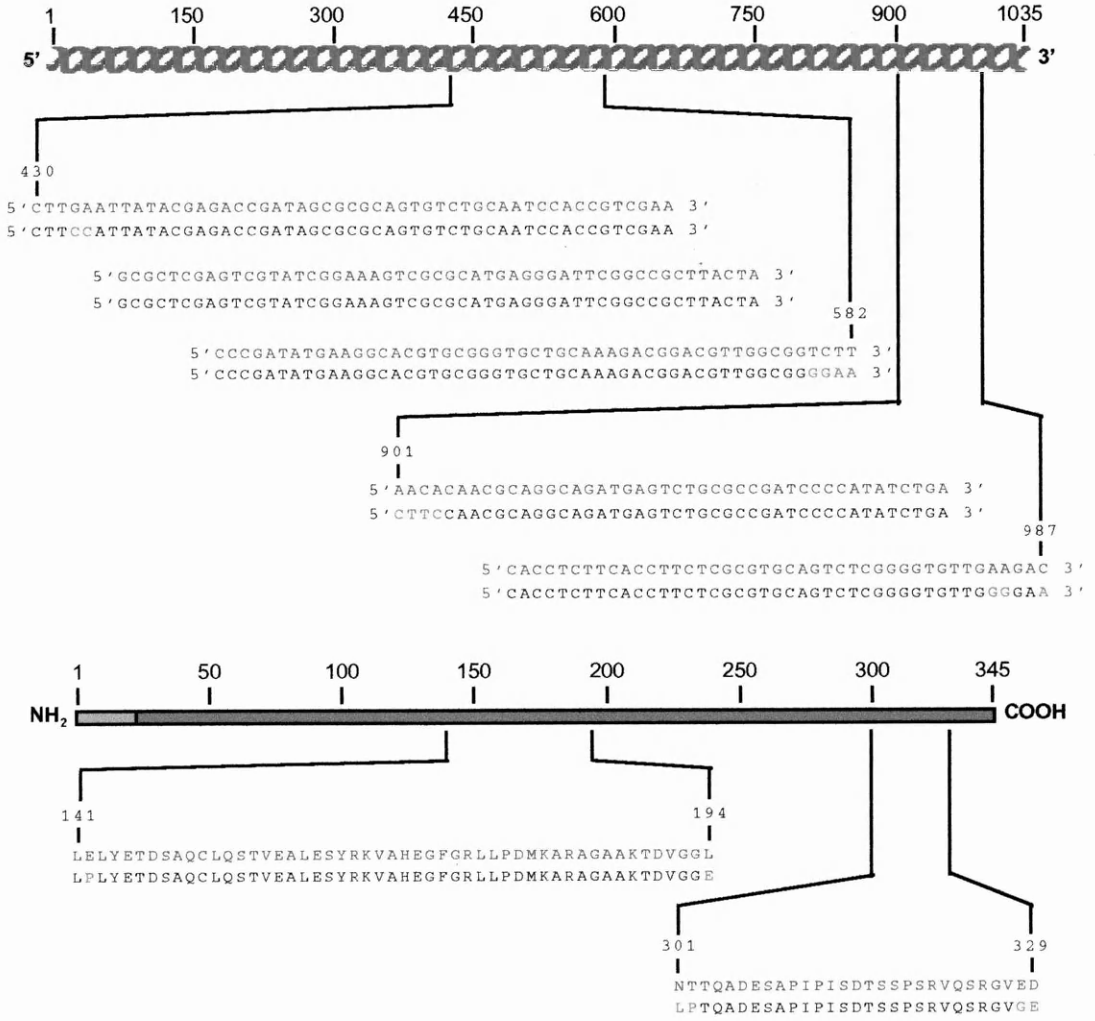


The clones selected from the fUSE2 library biopanned against anti-TpN44.5 showed two sizes of insert, so two of each were sequenced for comparison. Clones 17 and 23 were sequenced and found to contain the same 87 bp insert, while clones 18 and 24 were found to share the same 153 bp insert. Sequence comparisons using GCG showed the 87 bp insert to have homology with a portion of the *tpn*44.5 gene between base pairs 901 and 987. The larger insert was shown to have homology with the sequence between base pairs 430 and 582. Fig 68 shows the alignment of the

DNA sequences of the inserts with the database sequences. It also shows the deduced amino acid sequences of the inserts for comparison. The database sequence is shown in blue and the insert sequence in black, with non homologous residues highlighted in red.

Figure 69. DNA sequence of clone 17 and 24 from the anti-TpN44.5 enriched fUSE2 library.

DNA and deduced amino acid sequences of the inserts of clones 17 and 24 biopanned from the fUSE2 library using affinity purified anti-TpN44.5 antibodies. Database sequence is shown in blue and the insert sequence in black, with non homologous residues in red. The amino acid sequence is deduced from the DNA sequence.

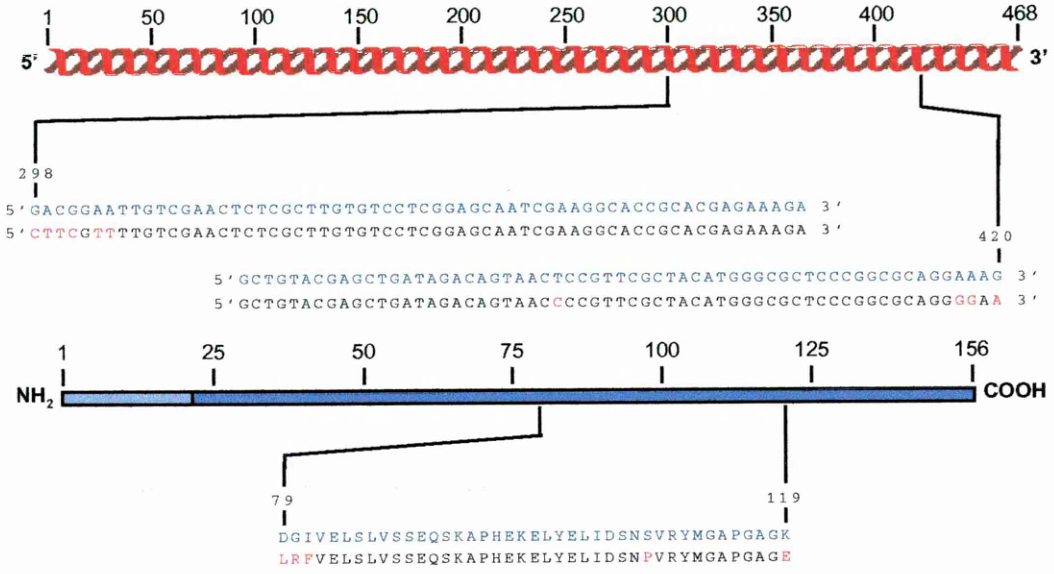


The clones selected from the fUSE2 library biopanned against anti-TpN17 all showed the same size insert, so two clones were sequenced. Clones 2 and 10 were found to contain the same 123 bp insert. This insert was found to have homology with the sequence of the *tpn*17 gene of *T.pallidum* between base pairs 298 and 420. Figure 70 shows the DNA and deduced amino acid sequences of

the insert aligned with the database sequences for comparison. The database sequence is shown in blue and the insert sequence in black, with non homologous residues highlighted in red.

Figure 70. DNA sequence of clone 10 from the anti-TpN17 enriched fUSE2 library.

DNA and deduced amino acid sequences of the insert of clone 10 biopanned from the fUSE2 library using affinity purified anti-TpN17 antibodies. Database sequence is shown in blue and the insert sequence in black, with non homologous residues in red. The amino acid sequence is deduced from the DNA sequence.



The clones selected from the fUSE2 library biopanned against affinity purified anti-TpN15 were previously shown to contain no insert, apart from clone 21 which did contain an inserted sequence. This clone was sequenced (data not shown) and confirmed to contain a 78 bp insert, but comparisons using GCG failed to find any homology with the sequence of the *tpn*15 gene at either the nucleotide or amino acid level.

Discussion

Synthetic peptide epitope mapping is a powerful tool for identifying simple linear epitopes, but is of limited use in defining conformational epitopes that require a degree of secondary structure for their expression. Phage display epitope mapping is a technique that can theoretically overcome this limitation by expressing larger protein fragments or even whole proteins on the surface of a bacteriophage.

The objective was to express fragments of the lipoproteins on the surface of bacteriophage M13 and enrich for binding motifs by biopanning with various anti-treponemal antibodies. The library must contain a reasonable representation of all the possible gene fragments for it to give an accurate picture of the reactive sequences present in the protein under study. The number of fragments of a certain size a gene can be split into can be calculated using the formula: (gene length-fragment size)+1. The total number of fragments possible is the sum of all those fragment lengths that are multiples of three, up to the length of the gene. Fragment lengths that are not multiples of three will cause a frame shift in the gene III sequence and will not be propagated. Not all fragments will represent the native sequence due to frame shifts allowing sequences in the two other reading frames to be expressed. For the purpose of the SurfZAP library constructions, TpN47 is 1302 base pairs long and can be split into 282317 unique clonable fragments. Similarly, TpN44.5, TpN17 and TpN15 can be split into 178365, 36426 and 29751 unique clonable fragments, respectively. It must be noted that the fragments can be cloned in both orientations, therefore doubling the number of possible fragments. The number of independent recombinants required in the library must be greater than these values, because sampling variation will lead to the inclusion of some sequences several times, and the exclusion of other sequences in a library of this size. Clarke and Carbon (1976) derived a formula that relates the probability (*P*) of including any DNA sequence in a random library of (*N*) independent recombinants, where *n* is the number of possible DNA sequences:

$$N = \frac{ln(1-P)}{ln\left(1-\frac{1}{n}\right)}$$

Therefore, for the SZ47 random gene fragment library, to achieve a 95% probability ($P = 0.95$) of including any particular fragment, the primary library must contain 1.7×10^6 independent recombinants:

$$N = \frac{ln(1-0.95)}{ln\left(1-\frac{1}{282317x2}\right)}$$
$$= 1.7x10^6$$

If the same calculation is performed for the other libraries, the SZ44 would require $1.1x10^6$ independent recombinants, the SZ17 library would require $2.2x10^5$, and the SZ15 library would require $1.8x10^5$ independent recombinants to have a 95% probability of containing any given sequence. Comparing these values to the actual numbers of unique phage generated in the primary libraries shows the SZ47 and SZ44 libraries to contain approximately half the required numbers of unique phage, while the SZ17 and SZ15 libraries contain more than enough phage to represent all possible fragments. The fUSE2 library was constructed to contain a restricted range of fragment sizes, from 51 to 351 base pairs long, but cover all four major lipoprotein genes. The reasoning described above applies equally well to this library, so a similar calculation shows there to be 245228 unique fragments which can be cloned in two orientations. This would require $1.5x10^6$ independent recombinants to have a 95% probability of representing all possible fragments within the library. The fUSE2 primary library contained around $1x10^6$ unique clones and so is fairly close to the size required to contain all possible fragments. The diversity of the libraries was also shown by the range of insert sizes seen by PCR amplification. However, not all the clones will contain useful native sequences due to frame shifts and compound ligations. The real diversity of the libraries will be much lower than the theoretical value, but even small libraries have been shown to produce meaningful results (Wang *et al.*, 1995).

The SurfZAP libraries were biopanned against purified IgG from a pool of serum from patients with high titre syphilis antibodies. The failure to achieve enrichment of the libraries and detect any binding clones by M13 immunoassay was probably due to the low specific activity of the immune human antibodies used for biopanning. In immune human sera the proportion of the total IgG that is disease specific is extremely small compared to even hyperimmunised animal serum. Most of the IgG is non-specific and results in the biopanning solid phase having a relatively poor binding capacity and a tendency for non-specific interactions. A large number of the binding phage will be bound non-specifically, resulting in poor enrichment of specific binding phage over background binding. Affinity purified antibodies were used to increase the specific activity of the solid phase used to biopan the phage display libraries and improve the enrichment of specific binders over background binding.

Even after enrichment with their appropriate affinity purified antibody, the SurfZAP libraries, SZ47, SZ44 and SZ17, failed to show any binding clones and the inserts all proved to be unrelated to the

lipoproteins. Only the SurfZAP library, SZ15, produced a clone that was reactive in the M13 immunoassay and contained a DNA sequence homologous to a region of the *tpn*15 gene between bases 121 and 375. The fUSE2 library, after biopanning with affinity purified antibodies specific for each of the lipoproteins produced binding phage homologous with regions of TpN47, TpN44.5 and TpN17. No binding phage were detected after enrichment with affinity purified anti-TpN15 antibody. The DNA inserts were shown to be homologous with regions between bases 748-888 of TpN47, bases 298-420 of TpN17. Two sequences were isolated from TpN44.5 homologous with the sequence between bases 430-582, and bases 901-987. Comparison of the sequences isolated from the phage display libraries with the reactive regions identified in the synthetic peptide epitope mapping studies shows a degree of concordance. The 255 bp SZ15 sequence covers a significant proportion of the TpN15 lipoprotein. Therefore, it is not surprising that it binds well to the solid phase as it contains most of the protein sequence and all but one of the reactive regions identified in the synthetic peptide epitope mapping studies. The sequences isolated from the fUSE2 library were shorter than the SZ15 insert, relative to the overall size of the parent protein. The enriched TpN47 sequence covered the region D, an intervening sequence plus the start of region E from the synthetic peptide studies. The TpN17 sequence that was isolated covered region A plus a contiguous sequence of amino acids that were non reactive in the synthetic peptide studies. The first of the two TpN44.5 sequences covered region A identified using synthetic peptides, and the second sequence corresponded to a region close to the C-terminus of the protein identified by only a few serum specimens and the affinity purified antibody.

Generally, the phage display technique isolated sequences that are antigenic, which is shown by their specific reactivity in the M13 immunoassay. They also broadly correspond to some of the sequences previously identified in the synthetic peptide epitope mapping studies. However, it is of note that the regions identified are all relatively long sequences compared to the reactive regions identified using the synthetic peptides. In phage display systems the inserted sequence is expressed as a gene III fusion and so has a tendency to interfere with cell attachment and hence propagation. Therefore, in the absence of any selection pressure on the phage population there would be a tendency for native phage or phage with small inserts to be preferentially propagated because their inserts have less effect on the function of the gene III protein. The preferential propagation of large gene III fusions in the presence of antibody means they must have significant

selection advantages over other phenotypes. Single epitopes could be expressed by very small gene III fusions. The absence of this type of fusion in the enriched libraries suggests that the most critical part of the antibody response is not described by simple linear epitopes. The propagation of large fusions suggests that either multiple epitopes or conformational epitopes form a significant portion of the antibody binding activity.

The biopanning experiments with the fUSE2 library show that the affinity purified antibodies are perfectly capable of enriching for binding motifs, so the failure to identify sequences from the SZ47, SZ44 and SZ17 libraries is surprising. The most likely reason is that the SurfZAP libraries were not as complex as the characterisation and phage titre data suggested. The failure to produce binding phage from three of the libraries, and the fourth producing a virtually full length clone, suggests that the library construction may not have been completely successful. The construction method, using a nested PCR technique and random primers, is rather complex and likely to produce a high proportion of non-specific phage containing irrelevant sequences. The size of the primary libraries and diversity of insert size may not represent different fragments of the lipoprotein genes, but nonsense sequences from primer-primer annealing. The libraries may be more like random peptide libraries rather than random gene fragment libraries. The fUSE2 library was constructed in a much simpler manner by fragmenting and cloning the genes themselves. Any recombinant phage produced should contain an insert at least derived from the target DNA. Although there is still scope to produce non-specific phage due to frame shifts and multiple inserts, this method should be more reliable. When the fUSE2 library was biopanned with several different specificity antibodies it produced sequences related to the source of the antibody, suggesting the library initially contained multiple phage phenotypes. However, to produce a single binding clone in each case was an unexpected result. It was initially thought that an antibody would select many different sequences, some related and sharing common sequences, across the protein.

Many researchers have investigated the factors affecting the efficiency of the phage display system. The library enrichment process has been examined using model systems to calculate the relative enrichment of various clones over non-binders (McCafferty *et al.*, 1990; Brietling *et al.*, 1991; Bass *et al.*, 1990; Barbas *et al.*, 1991). The general findings are that the number of phage binding and the enrichment of specific clones over background increases with affinity across the range. The enrichment of binding clones over non-binders can be as much as 40,000 fold or greater in a single

round of biopanning. Whereas the enrichment of high affinity over low affinity clones is more modest, a 4-fold increase in a 10 nM clone over a 320 nM clone. However, after three rounds of biopanning even the higher affinity clone would show a 64-fold increase over the low affinity clone. So, selecting 10-12 random clones from the enriched library is likely to yield repeated isolates of the same phage type.

The expectation that enrichment of the random gene fragment libraries would result in a range of different clones being selected, rather than just one, is probably unlikely. An alternative explanation might be that the libraries do not have the necessary complexity to express the full range of possible motifs. Only extensive sequencing of the primary library would answer this question. Alternatively, it could be a result of the biopanning protocol, which used a short binding time and a stringent wash protocol to remove unbound phage. This would have the tendency to select for high affinity clones by limiting the time available for binding, therefore favouring clones with high association rates. The original objective was to identify all the reactive regions within the protein and gain some idea of their relative potency by their representation in the enriched library. To accomplish this the biopanning protocol would have to allow a range of binding clones of different affinities to be selected. If the binding time were lengthened to allow the reaction to come closer to equilibrium and the wash protocol made less stringent, perhaps the range of specificities recovered would be increased. Most biopanning protocols are designed to facilitate the recovery of rare binder from the library, whereas in this study most of the clones should show some reactivity to the solid phase. A different strategy would be required to maximise the number of distinct binding clones recovered. Examination of the library after the first round of binding would probably provide a better idea of the range of binding clones present, rather than after several rounds of biopanning when the library may just represent the best binder. Perhaps phage display is unsuitable for providing this sort of qualitative information because the selection pressures produced by a few rounds of biopanning are so high that it will only really ever find the best binder in the library.

Epitope mapping using this approach requires a good understanding of the true complexity of the library under study. The diversity can only be assessed by extensive sequencing of the primary library to determine the range of inserts present. Gene targeted random epitope libraries are only of use for a single experiment which makes extensive sequencing of the library impractical. However, random peptide phage display libraries can be used for selection against many different targets and

so sequencing a selection of the primary library becomes a better investment of time. Also, with each subsequent experiment using the same primary library and the identification of new binding motifs, so confidence in the complexity of the library grows. This suggests that random peptide libraries might be a more appropriate approach from the point of confidence in diversity, but they do have other limitations. The best primary libraries are restricted to around 10^{10} individual clones, which limits them to only confidently displaying all combinations of seven amino acids. Libraries of longer sequences will be incomplete. This means that random peptide libraries are only suitable for identifying simple linear epitopes. They will fail in situations where significant antibody responses are directed towards conformational epitopes.

Without a detailed knowledge of the diversity of the libraries constructed here it is difficult to draw any firm conclusions as to the significance of the binding motifs identified. If the libraries genuinely possess a good representation of all possible binding clones, then it could be argued that the binding clones recovered represent immunodominant regions within the proteins. However, if the libraries lack diversity then these binding clones simply represent the most reactive regions represented in the libraries. Which ever interpretation is true, it is interesting to note that the binding clones all represent relatively large segments of the protein sequence. This suggests that simple linear epitopes are not the most significant antigenic determinants, and conformational epitopes probably represent the most significant binding motifs present. Since these long sequences almost certainly contain multiple epitopes, including some linear epitopes, this might enhance retention by the solid-phase by allowing multipoint attachment of the protein. So, the long sequences might be those with the highest affinity for the solid phase through a combination of interactions.

Phage display is a powerful means of selecting rare specific binders in a library from non binders or low affinity binding clones. Therefore, it has been very successful in determining the target sequence of monoclonal antibodies and in isolating single chain antibodies of a desired specificity. It has efficiently identified the highest affinity clones within the libraries specific for the anti-*T.pallidum* antisera. It is perhaps less well suited to mapping the spectrum of antibody epitopes within a protein.

Summary

The work described here provides useful information in two areas: the effectiveness and application of the phage display technique itself, and the sequences of the antigenic determinants within the lipoproteins of *T.pallidum*. The construction of the libraries is central to the success of the technique. The method used to construct the SurfZAP libraries was found to be too complicated to give confidence in the relevancy of library contents. The simpler technique used to construct the fUSE2 library produced greater confidence that the library contained relevant sequences. Phage display proved an effective technique for identifying high affinity binding motifs within the libraries, but it does not give any information on the range of epitopes present within the proteins. However, it provides a more complete picture than synthetic peptide epitope mapping as it is not restricted to linear epitopes representing only 10% of the response. The technique identified several long protein sequences that represented high affinity binding motifs, preferentially retained by the solid phase. It is difficult to deduce the exact nature of the epitopes responsible for their retention by the solid phase, but several possibilities exist. They might contain significant conformational epitopes, or a mixture of epitope types which produce high affinity binding through multipoint attachment to the solid phase. Clearly, the sequences identified have potential as antigens for use in diagnostic tests, but require further investigation.

Chapter 10

Synopsis

Conclusions

The antigens most universally reactive over the range of disease stages tested were, TpN47, TpN44.5, TpN17, and TpN15, which confirmed the literature and provided a sound platform for the subsequent work. The most significant conclusion drawn, and one not completely apparent from the literature, was the importance of TpN47 during early infections, being the only relevant specificity present in some patient's specimens. Additionally, there are no obvious patterns of antibody responses that are indicative of particular disease stages. These four antigens became the principal candidates for the production of recombinant proteins.

The production of recombinant proteins proved more problematic than expected in some cases. The full lipoprotein sequences of TpN44.5, TpN17, and TpN24-28, were well tolerated by the *E.coli* cells and expressed in moderate levels. Their expression produced different molecular weight forms of each recombinant protein which suggested putative processing, probably by lipid-modification, of these recombinant proteins was occurring. This processing was eliminated by deletion of the lipid-modification signal sequence. The full lipoprotein sequences of TpN15 and TpN47 proved difficult to clone, possibly due to toxicity of the recombinant proteins. They were finally successfully cloned as the mature protein sequences, minus their lipid-modification sequences, fused to vector-coded thioredoxin. These constructs were well tolerated and produced high levels of expression. The recombinant proteins were purified and all seemed to be antigenic as judged by their reactions with anti-*T.pallidum* antisera.

The antigenicity of the recombinant proteins was evaluated to identify any differences between their antigenicity and that of the native antigens tested earlier. TpN24-28, a minor antigen that appears as a diffuse band on SDS-PAGE, was found to be disease specific, but does not react with sufficient numbers of patient specimens to be a significant diagnostic antigen. The antibody responses to the other recombinant proteins, TpN47, TpN44.5, TpN17, and TpN15, showed an overall pattern similar to those seen with the comparable native antigens, suggesting that their antigenicity was similar to the native antigens. The frequency and strength of the responses suggested that all four recombinant proteins were good candidates, individually or in combination, for the development of an enzyme immunoassay for the serodiagnosis of syphilis. However, none of these antigens were universally reactive with all serologically confirmed cases of syphilis, so an

enzyme immunoassay for the detection of all stages of syphilis infection would probably require more than one antigen.

During the development of an enzyme immunoassay it was found that assays using single recombinant antigens lacked sensitivity, and the nature of the spread of the results suggested that they each missed key antibody responses that were detected by antigen from whole organisms. A combination of the four recombinant proteins produced an enzyme immunoassay with superior performance to that achieved by each of the recombinant antigens used individually. This was manifested as a greater difference between the absorbances of the positive and negative specimens, and a tighter correlation with the results of the native antigen based assay. It also showed better performance than the assay based on antigen derived from whole organisms that was used for comparison. Therefore, effective serodiagnosis was shown to require the use of a combination of four antigens to provide adequate sensitivity. The recombinant antigen-based enzyme immunoassay developed here has the potential to provide an alternative to assays based on antigen derived from *in vivo* cultured of whole organisms.

The sequences within the antigens responsible for their antigenicity was investigated using overlapping synthetic peptides complementary to the native amino acid sequence. The antibody responses were not found to be directed towards clearly defined sequences of linear epitopes within a background of low responses. A complicated picture of multiple responses across the entire sequence of the proteins was seen, however some relatively long consensus antigenic sequences were identified. This picture suggested that a high proportion of the diagnostically significant antibody responses were probably directed to discontinuous epitopes. Therefore, it is was thought unlikely that short synthetic peptides would be reactive enough to bind sufficient antibody to provide adequate serodiagnosis.

The antigenic sequences within the proteins were further investigated using phage display to examine a much wider variety of potential antibody binding motifs. Phage display proved an effective technique for identifying high affinity binding motifs, and recognised several long protein sequences within each antigen. The preferential selection of long protein sequences strongly suggests that simple linear epitopes do not represent the most significant binding motifs. A significant proportion of the antibody response is probably directed towards discontinuous epitopes. A conclusion that is supported by the evidence drawn from the earlier synthetic peptide work. The

binding observed might not be due to conformational epitopes, but could be a result of a mixture of epitope types which produce high affinity binding through multipoint attachment to the solid phase.

In conclusion, the antigenic activity of these proteins is not confined to discrete areas, but is spread across the whole protein. It is quite probable that a significant proportion of the antigenicity resides in conformational epitopes, which requires longer protein sequences for expression. Therefore, synthetic peptide antigens would be unsuitable as antigens for serodiagnosis. A combination of recombinant proteins remains the best means of providing the antigenicity required for adequate serodiagnosis of syphilis at this point.

Future work

The recombinant antigen-based enzyme immunoassay shows potential, but the preliminary evaluations performed here only confirm the feasibility of the design; more comprehensive testing would be required to fully validate the design. The solid phase was optimised using a single pool of syphilitic serum, so the coating concentrations should be further refined to ensure the best compromise of sensitivity at all stages of the disease. Testing with a wider variety of specimens would better evaluate the sensitivity and specificity, particularly potentially cross reactive specimens, and difficult specimens such as those from patients co-infected with HIV. This testing should also consider the concerns over the inclusion of TpN44.5 in the antigen mixture. It should be determined if the inclusion of TpN44.5 does cause false positive reactions, or if its omission would be detrimental to assay performance. Once the assay design was finalised, the remaining tasks would be to determine a cut-off value, and ensure the reproducibility and robustness of the assay.

The synthetic peptide and phage display epitope mapping studies identified antigenic sequences that should be investigated further to determine if they are suitable as diagnostic antigens, either singly or in combination. This would be best pursued by constructing a range of artificial recombinant proteins from the sequences and using them to test the diagnostic efficacy of the polypeptides produced. Further testing of the SurfZAP libraries is unlikely to yield much extra information because they are too incomplete, but there would be benefits from biopanning the fUSE2 library against other syphilitic antibody preparations. Comparison of any sequences selected with those previously isolated might reveal more information on the nature of the antigenicity of these antigens. It would also improve confidence in the complexity of the library and the validity of any conclusions drawn.

A simple synthetic antigen would offer many potential benefits. It would significantly reduce the costs of syphilis testing compared to current treponemal tests using native antigen, and also allow simple assay designs, more suitable for use in developing areas, to be adopted. This may promote the introduction of screening and treatment programmes in the poorer areas of the world where syphilis is still a significant problem, closely associated with the spread of HIV.

Chapter 11

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